Characterizing G-Quadruplex Mediated Regulation of the Amyloid Precursor Protein Expression

By

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October, 2016

A Dissertation Presented to the Faculty of

Drexel University Department of Biology

In partial fulfillment of the Requirements for the Degree of Ph.D.

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Acknowledgements

Proverbs 3:5-6, “Trust in the Lord with all thine heart and lean not unto thine own understanding. In all thine ways acknowledge Him and He shall direct thy paths” I want to start off my acknowledging my LORD, who is known by many names. He is El Elyon, The Most High God; He is YAHWEH Shammah, for He has been there for me; He is YAHWEH Yireh, for He has provided for all of my needs; He is YAHWEH Shalom, for He is my peace. He is Yeshua Ha Mashiach, for He is my Savior. Coming from humble beginnings, my faith in the LORD has been the source of strength and encouragement to overcome the many obstacles I faced growing up, as well as in my pursuit for my Ph.D.

I’d like to thank my advisor, Dr. Aleister Saunders, for bringing me into his lab and for his help and support during the past 5 years. I know I had struggles in the lab as far as research, but I am thankful to him for giving me an opportunity to grow and learn as a graduate student and as young scientist. I am especially thankful that Aleister allowed me to explore other opportunities outside of the lab such as teaching both locally and internationally, research conferences, additional courses, and professional development workshops. I want to thank my co-advisor, Dr. Michael Akins who has been very instrumental in moving my thesis work forward. Michael brought me into his lab as a joint student and I was able to learn various techniques from members in his lab that really help me in my own research. I appreciate the time he took to mentor me and just for his enthusiasm to advance scientific research. I’d like to thank my thesis committee Drs. Daniel Marenda, Elias Spiliotis, Tali Gidalevitz, and Jeff Twiss for their guidance and helpful suggestions related to my research, as well as in courses and seminars.

Next, I want to thank Drs. Haizhi Wang and Michal Sharoni for their help and support. I really felt that we were a small lab family. We struggled and celebrated together and created an atmosphere that was still fun and exciting to conduct research. It has meant so much to me for them to take the time out of their busy schedules to teach me a technique or listen to me give a search talk and provide me with feedback that didn't make me feel like I was not qualified to be a graduate student. I also want to thank the previous lab members Drs. Suruchi Utreja and Anna Vorobyeva for helping me when I initially joined the lab, as well as Brendan Mullen, Angela Tuckowski and Gavi Horwitz for their help.

Finally, I'd like to thank my wife, Elisha Crenshaw and our children, Israel, Zachariah, and Elaya for their continued love and support. It meant so much to me to come home to smiles and encouragement from them. When I felt like giving up or that I wasn't qualified to pursue a doctoral degree, they believed in me, prayed for me, as well as laughed and cried with me. I am grateful because now our children can grow up and realize that they too can follow their dreams.

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Abstract

A central event in Alzheimer's disease is the accumulation of amyloid β (Aβ) peptides generated by the proteolytic cleavage of the amyloid precursor protein (APP). APP overexpression leads to increased Aβ generation and Alzheimer's disease in humans and altered neuronal migration and increased long term depression in mice. Conversely, reduction of APP expression results in a decreased Aβ levels in mice as well as impaired learning and memory and decreased numbers of dendritic spines. Together these findings indicate that therapeutic interventions that aim to restore APP and Aβ levels must do so within an ideal range. To better understand the effects of modulating APP levels, we explored the mechanisms regulating APP expression focusing on post-transcriptional regulation. Such regulation can be mediated by RNA regulatory elements such as guanine quadruplexes (G-quadruplexes), non-canonical structured RNA motifs that affect RNA stability and translation. Presented in this thesis, we identified the G-quadruplex as a novel endogenous regulator of APP expression within the APP mRNA in its 3'untranslated region at residues 3008-3027 (NM_201414.2). This sequence exhibited characteristics of a parallel G-quadruplex structure as revealed by circular dichroism spectrophotometry. Further, as with other G-quadruplexes, the formation of this structure was dependent on the presence of potassium concentration. Moreover, we present preliminary data demonstrating that FMRP and FXR2P bind to the APP G-quadruplex sequence and regulates it expression.

Chapter 1: Introduction

BACKGROUND AND SIGNIFICANCE

The first case of Alzheimer's disease was identified more than 100 years ago and it would takes decades more for it to be recognized as the leading cause of the dementia [1]. Alzheimer's disease (AD) is a progressive neurodegenerative disease that is the most common form of dementia [2]. Dementia is a decline in memory, language, and cognitive functions that affects an individual's capacity to perform daily activities or routines [3]. People living with AD may begin to have difficulty remembering names or new information and may experience depression. As the disease progresses, individuals experience impaired judgment, disorientation, changes in behavior or mood, as well as difficulty in swallowing, speaking, and walking [4].

Risk Factors for AD

Age is one of the best known risk factors for AD. It is estimated to be ~5.4 million Americans living with AD, approximately 5.2 million are 65 years of age or older. [5]. There are two categories of age related onset of AD which are early and late onset. Early onset AD occurs before the age of 65 and accounts for 1-6% of AD cases [6]. Research have mapped mutations on chromosome 21 that encode the Amyloid Precursor Protein (APP) [7-9] as well as Presenilin 1 (located on chromosome 14) [10] and Presenilin 2 (located on chromosome 1) [11]. There is evidence demonstrating that non-memory symptoms, such as language impairment, are more prevalent in early onset AD as well as a faster progression of cognitive decline and function [12]. The second category of age related AD is late onset, which is the most common, occurring at age 65 and older in about

90% of AD cases [13]. Mutations in APOE gene accounted for ~27% of late onset AD [14, 15]. Advances in genome wide association studies have identified mutations in at least 20 genes involved in late onset AD [16].

Heredity can be a risk factor of AD. Individuals who may have had a parent or sibling with AD are at a higher risk of developing this disease [17]; especially if genes containing mutations are inherited, such as in APOE. Environmental factors such as lack of physical activity, cardiovascular disease, and diet can contribute to AD. Lastly, individuals who have repeated occurrences of traumatic brain injury are at a higher risk of developing AD and other dementias [18].

The Brain and AD

In healthy individuals, the brain contains approximately 100 billion neurons, which form structures known as synapses whose function is to communicate with each other chemically by the release and uptake of neurotransmitters at the synapse. There are approximately 100 trillion synapses formed between the neurons in the brain. In AD, those neuronal functions are altered. Brains of AD patients exhibit severe brain atrophy compared to brains of healthy individuals [19]. The reduction in the size of the brain is attributed to the loss of neurons and synapses which leads to the memory loss and decline of cognitive functions. At the molecular level, AD is characterized by the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles. Aβ plaques are derived from proteolytic cleavage of amyloid precursor protein (APP) [20] (**Figure 1.1**). [21]). Aβ peptides begin as oligomers and can aggregate extracellularly in neurons leading to the deposition of Aβ plaques [22].The neurofibrillary tangles arise due to the hyper-phosphorylation of the tau protein, which is a microtubule stabilizing protein, and

aggregates inside the neuron [23]. While the presence of Aβ plaques or neurofibrillary tangles are characteristic hallmarks of AD, there has been much debate on which one is the main culprit in AD pathogenesis [24, 25].

Amyloid Hypothesis

The amyloid hypothesis states that deposition of Aβ is the initial contributor to AD pathogenesis and that Aβ leads to the formation of neurofibrillary tangles and neuronal loss [26]. There is a wide range of support for the amyloid hypothesis. Oligomeric $A\beta_{42}$ from AD brains is sufficient to induce synapse density, impair memory in rodents, and induce tau hyper-phosphorylation [27]. Furthermore, support for the amyloid hypothesis showing that mutations in in the genes that cause early onset AD increase Aβ levels [27].

Treatment for AD

While there is no cure for AD, the amyloid hypothesis has driven exploration of AD drug therapies that focus on targeting Aβ, APP, or Presenillin1/2 [28] due to the fact that mutations in these genes lead to increase Aβ and early onset AD. One strategy for developing AD drugs is to target the proteases that cleave APP to generate Aβ. Those proteases are beta secretase and gamma secretase. β-secretase is the initial protease to cleave APP to generate Aβ [29, 30]. Drugs that inhibit β-secretase have been shown to reduce Aβ levels the in cerebral spinal fluid of AD patients [31]. The drawback from using inhibitors of β-secretase is that this protease has other protein substrates that have an important role in myelination and sodium homeostasis [32, 33]. Additionally, β-secretase knockout mice have higher mortality rates as well as memory impairment [34]. γ- secretase inhibitors have also been investigated as potential therapeutic targets. Cleavage by γ secretase is the final step in releasing Aβ from APP [35]. In principle,

inhibiting γ secretase would be a reasonable strategy to limit the production of Aβ. However, as with beta secretase inhibitors, γ secretase has additional substrates, such as Notch, which is essential for cell growth and differentiation [31, 36]. Recent clinical trials for γ-secretase inhibitors indicated severe negative side effects which were failure to slow the progression of AD and improve cognitive function [37, 38].

A number of drugs have been designed to aid in Aβ clearance [39, 40]. This approach has been met with some challenges due to the fact that there is a need to identify biomarkers for AD in its early stages [41]. By the time AD symptoms are present, individuals could already be in the advanced stages of AD that may render this approach ineffective. Further research is needed to investigate drugs that target either beta or gamma secretase to modulate their levels specifically for APP, without causing deleterious effects on their other substrates. A complementary strategy that avoids off-target effects that come from manipulating the secretases would be to directly modulate APP levels, since overexpression of APP contributes to AD pathology. It will be informative to explore regulatory elements that enhance or suppress APP levels to shed light on mechanisms regulating APP.

The Biology of APP

The gene encoding APP is located on chromosome 21 and alternative splicing produces three major isoforms – APP695, APP751, and APP770 that vary in their amino acid length [42]. APP751 and APP770 are expressed in most tissues whereas APP695 is predominantly in neurons [6, 42]. APP is a type I transmembrane protein that spans across the plasma membrane having the N-terminal region oriented towards the extracellular matrix while the C-terminal region is cytosolic [43]. APP contains a signal

peptide sequence and once it is synthesized in the endoplasmic reticulum and undergoes fast axonal transport to the plasma membrane [44] where it can be cleaved by proteases which release its extracellular, intracellular, and intramembrane domains [45].

Function of APP

While APP has been extensively researched over the past few decades, the function of this protein is poorly understood. APP has been implicated in synapse formation, axonal migration, and synaptic plasticity [46]. Studies from APP knockout mice indicated that the mice had neurological deficiencies including grip strength and locomotor activity [47], as well as alter synaptic function and spine density as well as a reduction in spatial memory [48]. Additionally, upon examining overexpression of APP mice also showed defects in memory, altered synapse, and decrease in proteins responsible for synaptic plasticity [49].

It has been previously reported that APP could function as a cell surface receptor due to the similarities in processing with Notch [50, 51]. It was previously reported that Aβ could bind to APP suggesting that it could serve as a ligand for APP [52]. APP has also been shown to interact with extracellular matrix proteins such as heparin and laminin suggesting that APP could function as a cell adhesion molecule [53]. Additional evidence supporting a role for APP in cell adhesion comes from studies showing that APP can dimerize and that dimerization of APP stimulates the formation of specialized, presynaptic compartments in neuronal co-culture assays [54]. One of the most compelling arguments for the function of APP is in neurite outgrowth and axonal migration [55, 56]

Proteolytic Processing of APP

APP cleavage is an orchestrated process that requires several sequential cleavage steps that occur in regulated intramembrane proteolysis (RIP). The RIP process enables the cells to respond to their extracellular environment by allowing the cleavage products to serve as signaling molecules that aid in a variety of biological processes. The first cleavage results the release of the protein's ectodomain while the second cleavage occurs within the transmembrane domain releasing the protein's intracellular domain [57, 58]. The RIP process of APP begins with cleavage by alpha secretase or beta secretase which occurs by two pathways that either precludes Aβ generation (termed Non-Amyloidogenic pathway) and one leads to the generation of Aβ (Amyloidogenic pathway) [58]. In the non amyloidogenic pathway, cleavage by alpha secretase occurs within the Aβ domain and releases the sAPPα ectodomain. Subsequent cleavage by gamma secretase releases a shortened Aβ peptide known as p3 and AICD. The amyloidogenic pathway begins with the cleavage of APP by beta secretase which releases sAPPβ ectodomain. APP is then cleaved by gamma secretase, where it releases AICD and Aβ (Figure 1.2 [59]) [58].

Function of APP Cleavage Products

The roles of the APP proteolytic products are poorly understood; however, investigations into their biological roles have been investigated. Initial cleavage of APP in the non-amyloidogenic pathway by α-secretase releases sAPPα and a C-terminal fragment (CTF-83). sAPPα appears to have more positive effects such as neuroprotective roles against oxygen/glucose deficiency through the inhibition of calcium currents and activating potassium currents [60, 61]. sAPPα also functions to promote neurite outgrowth and synaptogenesis [62]. There are no known biological functions for CTF-83. Cleavage by α and γ secretases release p3 which doesn't have a clear biological function. In the

amyloidogenic pathway, initial cleavage of APP by β-secretase releases sAPPβ and CTF 99. Neuroprotective functions are not attributed to sAPPβ. sAPPβ was demonstrated to be involved in axonal pruning by serving as a ligand for the death receptor 6 (DR6) and triggering apoptosis [63]. CTF 99 has not been shown to have a biological function. Cleavage of APP by β and γ secretases release the Aβ domain. Extensive research has been geared towards studying the toxic ramifications of Aβ accumulation, however, Aβ seems to have a relevant biological function. In small concentrations, Aβ was shown to depress neuronal activity [64], which is beneficial to prevent excitotoxicity. The last fragment, which is common to both non-amyloidogenic and amyloidogenic pathways, is the APP intracellular domain (AICD) that is released upon cleavage of CTF 83 or 99 by γ-secretase, producing a 57 or 59 amino acid sequence AICD. AICD acts as a signaling molecule that binds to Fe65 which then recruits the histone deacetylase Tip60 and together they translocate the nucleus to activate the transcription of several genes [65-67]

Regulation of APP Gene Expression

The levels of Aβ as well as the proteases responsible for its generation play a pivotal role in Alzheimer's disease pathogenesis. While the regulation of the various genes involved in AD may be important, it may be just as important to understand how the expression of APP is regulated. Since APP is a substrate for beta and gamma secretase, the amount of Aβ generated from proteolysis of APP will depend on how much of the substrate is available for cleavage through the amyloidogenic pathway [68]. Increased expression of APP correlates with an increase in the production of Aβ. Evidence for APP overexpression in generating more Aβ comes from individuals with trisomy 21 who have higher amounts of APP mRNA when compared to control individuals [69, 70]. Mutations

in APP can also lead to increased protein levels and Aβ [71]. Additionally, certain regions of the brain contain more APP mRNA in AD patients than controls [72]. Therefore, it is important to understand the mechanism by which APP gene expression is regulated due to the fact that controlling the regulation of APP can also regulate the production of Aβ. To understand the mechanisms involved in regulating APP gene expression, we can examine transcriptional, post-transcriptional, and post-translational regulation of APP.

Transcriptional Regulation of APP

In order to investigate transcriptional control of APP, research has focused on the promoter region of APP. The promoter region of a gene contains cis-regulatory elements that enable the binding of proteins such as polymerases as well as transcription binding factors to promote or suppress transcription of a gene, thereby controlling the amount of protein being produced [73, 74]. Transcriptional analysis of APP revealed that the promotor region for the APP gene lacked a canonical TATA box region, but did contain a GC-rich region [74]. This GC-rich region is used to facilitate the binding of putative transcription factors [74]. Several transcription factors have been identified that bind to the APP promoter sequence. One transcription factor is the CCCTC binding factor (CTCF). This protein binds to a variety of DNA sequences and is also a regulator of c-Myc which is involved in apoptosis [75]. CTCF has been shown to bind to a particular domain proximal to the APP domain designated as ABPβ. Binding of CTCF to ABP(beta) activates the transcription of APP [75]. Other transcription factors such as HSF1, which in response to stress, activates APP expression [76]; the NK-kB/Rel family has been shown to regulate APP in neuronal cells [77]. The transcription factor SP1 has

been shown to bind to the promoter of APP and the overlaps with CA1 transcription factor [78]. The promoter region of APP is conserved from rodents to primates with all having the absence of the TATA Box and all containing the GC-rich sequence, suggesting an important biological function between the promoter regions and the transcription factors that bind [74, 79]. This may be of importance considering that if mutations are found within the promote sequences, they could influence the binding of transcription factors that may increase transcription. A 2006 mutational analysis of the APP promoter region large study conducted on Belgian patients revealed six genetic variations within the promoter region of APP in which three of the variants lead to a 2-fold increase in the transcription of APP [80]. In addition to the promoter region of APP influencing upregulation of the APP transcript, it has been previously shown that APP transcription can be influenced by local inflammation at sites of injury and was supported by research demonstrating that Interleukin 1, a pro-inflammatory cytokine, is secreted in response to brain injury and increases APP levels [81]. Further support of inflammation having an impact on APP mRNA levels comes from studies evaluating the effects of traumatic brain injury, a known risk factor for AD [82]. In one report, it was found that APP mRNA levels are upregulated in response to traumatic brain injury within 15 minutes [83]. Additionally, genes and proteins involved in regulating APP mRNA levels are also increased following traumatic brain injury [84].

Post-transcriptional Regulation of APP

Once the APP mRNA is transcribed, it can post-transcriptionally regulated. This process is usually regulated by sequences found within the transcripts 5’ untranslated region (UTR), coding sequence, or 3’untranslated region. These sequences enable the binding of

RNA binding Proteins (RBPs) where they can either promote or suppress translation [85]. In the 5' UTR, there are several regulatory elements that have been shown to regulate APP levels and influence its translation such as an internal ribosomal entry site (IRES), and iron response element (IRE), and an IL-1 enhancer response element [86-88]. Regulation of APP by the IRES was considered to regulate APP levels due to the fact that inhibition of the mTOR pathway, which is necessary for cap-dependent translation, still lead to an increase in APP levels [86]. This data lead to the finding of an IRES in 5' 50 nt sequence in the APP 5'UTR which enable translation of APP via a cap-independent mechanism [86]. It was reported previously that APP contains an IRE stem loop that was similar the one, which in response to intracellular iron levels, regulates L and H ferritin mRNA [89]. IRP1 was found to bind to the IRE found in the 5'UTR of APP, where in the absence of iron, prevents cap-dependent translation of APP by blocking the 40S ribosome at the 5' cap [89]. Interestingly, increasing iron concentrations influences APP mRNA translation by the IRES found in the APP 5'UTR. It remains unclear how the regulation of iron, or perhaps other factors, contribute to cap-dependent or cap-independent translation of APP mRNA. Lastly, the 5'UTR of APP contains a 90 nt region that interacts with IL-1, implicating a role for inflammation in regulating APP levels, which does not affect APP mRNA stability, but does regulate its translation [87].

The coding region of APP has been shown to be post-transcriptionally regulated by RNA binding proteins which influence its translation [90]. The Fragile-X Mental Retardation Protein (FMRP) has been predicted to bind to APP and act as a negative regulator of APP translation [91]. FMRP is highly expressed in the brain and mutations in the gene that encodes FMRP, *FMR1*, leads to reduction of the FMRP and causes Fragile X Syndrome.

Cognitive impairment is one of the major effects resulting from reduction of FMRP, which implicates this protein is necessary for normal cognitive function [92]. FMRP contains 2 major domains in which it interacts with its target mRNAs which are the KH domains, and second domain is a C-terminal RGG Box domain, which is a region rich in arginine and glycine repeats, is believed to interact with its mRNAs harboring guanine quadruplex structures [91-93]. High throughput sequencing methods such as HITs-Clip and PAR CLIP identified APP mRNA as a direct target for FMRP [94, 95]. Westmark and colleagues demonstrated that FMRP associates with APP mRNA through the coding region and negatively regulates its translation [96]. Additionally, knockdown of FMRP increased APP levels and Aβ levels. Additionally, stimulation with the mGluR agonist DGPH, rapidly increased APP mRNA translation [96, 97]. Another study demonstrated that FMRP associates with the coding region of the APP mRNA in Processing Bodies (P-Bodies), which are cytoplasmic foci that are sites for mRNA suppression through mRNA nonsense mediated decay or microRNA induced silencing, where APP mRNA is translationally suppressed [98]. FMRP levels decline with age suggesting that FMRP transcripts would be elevated. This is concerning considering that age is a major risk factor for AD and that APP overexpression can lead to increased Aβ production. Another RBP shown to bind to the APP mRNA coding sequence is Heterogeneous Nuclear Ribonuclear Protein C (HNRNP C). This protein belongs to a larger family of proteins that associate with pre-mRNAs in the nucleus and affect mRNA processing, metabolism, and transport [99]. This protein was shown to compete with FMRP for the binding site in the coding sequence where it promotes APP mRNA translation [98].

The 3'UTR of APP is ~1.2 kb in length and is conserved in all APP isoforms. Several regulatory elements have been identified in this region to regulate APP levels. Approximately 200 nucleotides downstream of the stop codon is a 29 nucleotide sequence that is suggested to de-stabilize APP mRNA and is the target sequence for Nucleolin [100]. Nucleolin is a major nucleolar protein and it is involved in chromatin decondensation, pre-rRNA transcription and ribosome assembly, and may have roles mRNA processing [101]. With the first 52 nucleotides of the APP 3'UTR is a region that stabilizes APP mRNA and is the binding site for six proteins, Rck/p54, plasminogen activator inhibitor-RNA binding protein 1 (PAI/RBP1), Y-box binding protein 1 (YB1), autoantigen La/Sjogren syndrome antigen B (La/SS-B) and elongation factor 1α (EF1a) [90]. It isn't fully understood if these proteins operate together to regulate APP or what roles these proteins have individually in regulating APP with the exception of Rck/p54. Rck/p54 overexpression was shown to increase APP levels [102], presumably by using its helicase activity to unwind this region, making it more accessible to additional factors which stabilize APP mRNA. The next regulators of APP mRNA through its 3'UTR are microRNAs. miRNAs are small, endogenous RNAs that negatively regulate mRNA by binding to complementary sequences primarily in the 3'UTR of its target mRNAs [103, 104]. There are two methods of negative regulation of gene expression by miRNAs depending on how they bind to their targets. One method is by binding with perfect complementarity to the sequence of the mRNA [105]. When this happens, the mRNA is degraded. The other method is by binding with near-perfect complementarity to the mRNA which enables the mRNA to be translationally suppressed [105]. Several miRNAs have been identified to bind to the 3'UTR and negatively regulate APP levels [106-108].

Furthermore, it has been demonstrated that miRNAs are downregulated in AD [109-111]. Furthermore, single nucleotide polymorphisms (SNPs) have been identified in miRNA binding sites which alter miRNA regulation [112]. This suggest dysregulation of APP translation via miRNAs can contributed to higher APP levels, and can invariably lead to AD. More recently, a guanine rich region in the 3'UTR of APP was shown to adopt a secondary structure known as a guanine quadruplex (G-quadruplex) and was reported to negatively regulate APP levels [113]. Guanine rich nucleic acids can interact with one another through Hoogsteen-Hydrogen Bonding and are further stabilized by monovalent cations such as potassium [114, 115]. The data demonstrated that mutations in the G-quadruplex region increased APP levels as well as Aβ levels [113]. The details of this regulation are mention in chapter 2.

Post-translational Modification of APP

Post-translational modification is the process of altering proteins once they have been translated. Some examples include APP is known to have several modifications such as N- and O- glycosylation, phosphorylation, and sialyation for example [45, 116, 117]. APP undergoes the constitutive secretory vesicle pathway in that once synthesized, it is transported to the endoplasmic reticulum (ER), then to the Golgi, and then to the plasma membrane before it's internalized and trafficked to the endosome or lysosome [45]. APP exists as immature APP (iAPP) and during its transport from the ER and Golgi, APP is post-translationally modified by N and O- glycosylation and becomes mature APP (mAPP) [118]. N-glycosylation of iAPP takes place in the ER and O-glycosylation in the Golgi. After N and O-glycosylation, mAPP is secreted to the plasma membrane [118]. Proper glycosylation is needed in order to obtain proper axonal sorting and metabolism of

APP [119] (Figure 1.3 [117]). Studies have indicated that APP contains two sites for N-glycosylation at Asn_{467} and Asn_{496} [120] and mutations of these sites prevents APP maturation and transport to the plasma membrane by retaining APP to the Golgi; leading APP to encounter Beta and gamma secretase to generate Aβ [121]. Additionally, APP bearing the Swedish and London mutations was shown to give rise to altered N-glycosylation that contained high amounts of bisecting GlcNAc residues [122]. Several O-glycosylation sites have been identified in APP. These sites are in found on Ser and Thr residues and functions remains poorly understood [123]. O-glycosylation gives rise to mAPP and proteolytic cleavage of APP occurs preferentially after O-glycosylation [118]. It was reported that a mutation from the amino acid Leu to Pro was effective in inhibiting O-glycosylation of APP and in doing so, decreased the amount of mAPP [118]. Although this region is not usually a site for O-glycosylation [124], it is suspected this mutation could lead to improper folding of APP and could potentially prevent APP from being secreted and confined to the Golgi where the endocytic environment increases the change of APP cleavage by beta secretase [125-127]. Phosphorylation of APP has been shown to occur at Thr_{668} which allows for beta secretase cleavage of APP, but prevents gamma secretase, thus reducing Aβ [128].

Summary

APP plays a pivotal in AD as proteolytic cleavage of this protein and overexpression are critical in the generation of Aβ. Given the lack of early indicators for AD and challenges facing the development of AD drugs, it is important to understand the mechanisms underlying regulation of APP gene expression and processing. Highlighted in this chapter are various regulatory elements that mediate APP expression and metabolism. Sequences

found within APP are capable of regulating its gene expression by promoting or suppressing transcription and translation of APP mRNA as well as serve as sites for post-translational modifications. Mutations in APP can increase APP expression, alter cleavage and post-translational modification, and alter APP trafficking, all of which can increase Aβ levels. These data indicate the potential to uncover new pathways involved in mediating APP regulation and processing in efforts to develop new targets for AD treatment.

Figure Legends

1.1 [21] (modified) Pathology of Alzheimer disease: (A) The two hallmark features of Alzheimer disease, β-amyloid plaques (arrowheads) and neurofibrillary tangles (arrows) in AD brain are revealed by the Bielschowsky silver stain.

1.2 [59] **(a**) Two pathways (β/γ and α/γ) of APP proteolysis. APP can be cleaved by either β- or α-secretase, which is then followed by γ-secretase cleavage. The designation of substrates and products are depicted.

1.3 [117] (modified): Schematic overview of the roles of glycosylation in the processing of APP. Correct glycosylation is required for axonal sorting and processing of APP. An alteration in N-glycosylation results in protein accumulation within the perinuclear region of the cell (1). Inhibition of the formation of N-glycans or complex N-glycosylation or sialylation of APP interferes with axonal sorting (2) and secretion (3) of APP, as well as the secretion of sAPPa (4), sAPPb (5), and Ab (6).

Figure 1.1

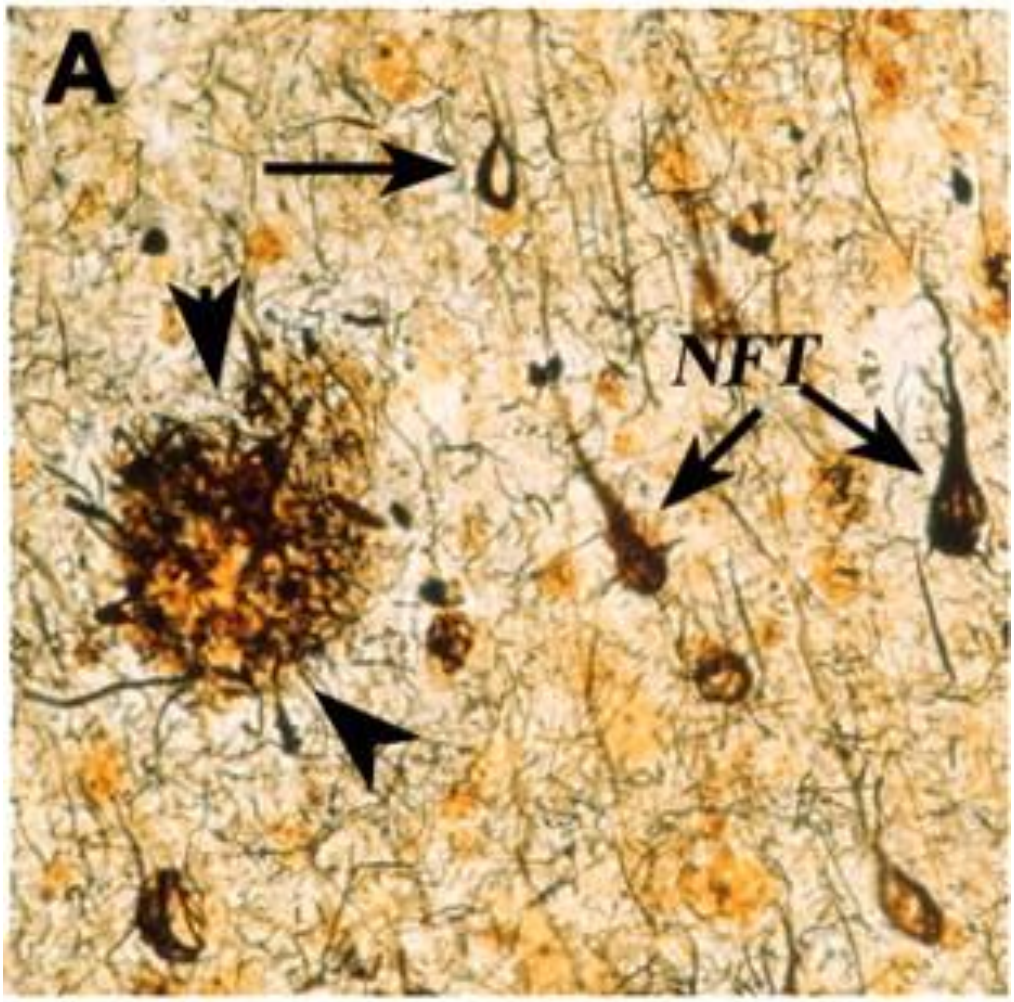


Figure 1.2

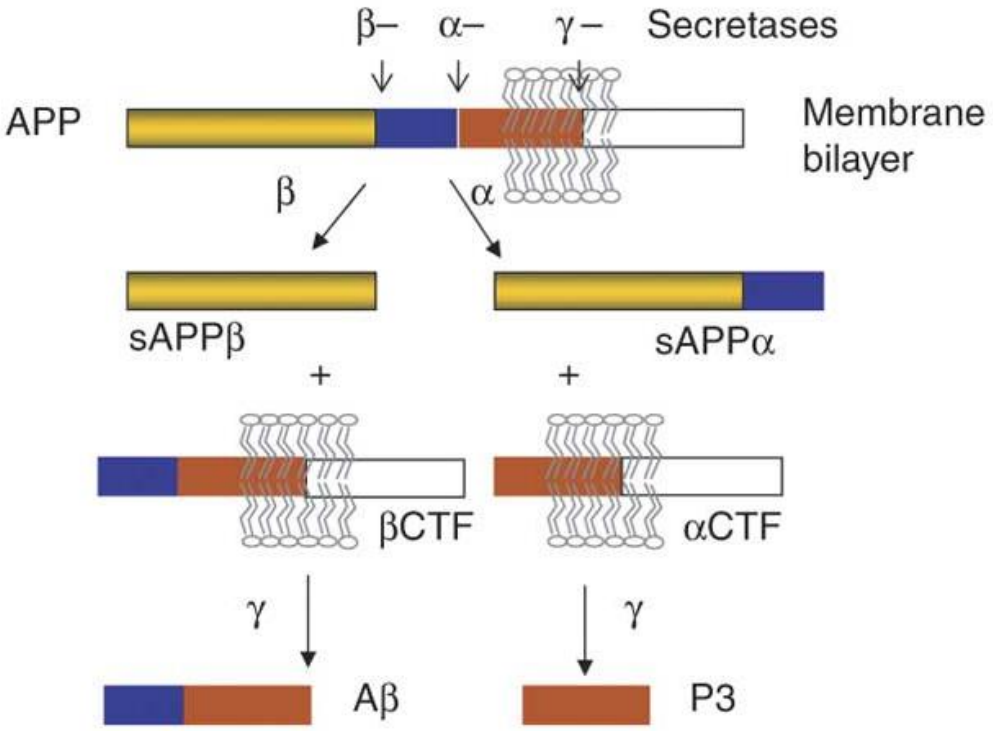
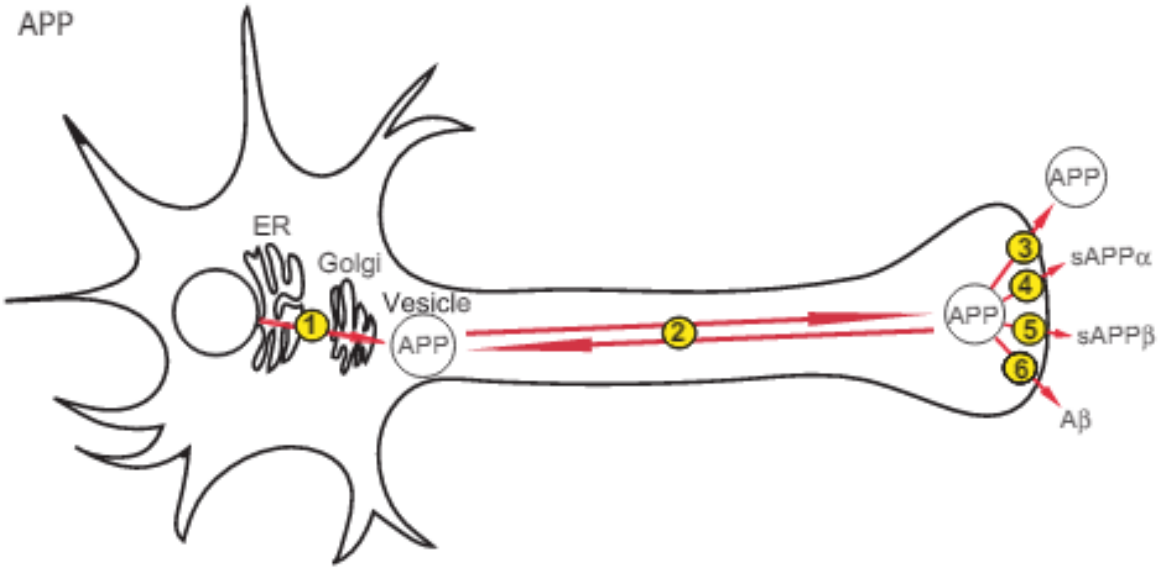

β– α– γ– Secretases
APP
Membrane bilayer
β
α
sAPPβ
sAPPα
+
+
βCTF
αCTF
γ
γ
Aβ
P3

Figure 1.3



Chapter 2

Amyloid Precursor Protein translation is regulated by a 3'UTR Guanine Quadruplex

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Abstract

A central event in Alzheimer's disease is the accumulation of amyloid β (Aβ) peptides generated by the proteolytic cleavage of the amyloid precursor protein (APP). APP overexpression leads to increased Aβ generation and Alzheimer's disease in humans and altered neuronal migration and increased long term depression in mice. Conversely, reduction of APP expression results in a decreased Aβ levels in mice as well as impaired learning and memory and decreased numbers of dendritic spines. Together these findings indicate that therapeutic interventions that aim to restore APP and Aβ levels must do so within an ideal range. To better understand the effects of modulating APP levels, we explored the mechanisms regulating APP expression focusing on post-transcriptional regulation. Such regulation can be mediated by RNA regulatory elements such as guanine quadruplexes (G-quadruplexes), non-canonical structured RNA motifs that affect RNA stability and translation. Via a bioinformatics approach, we identified a candidate G-quadruplex within the APP mRNA in its 3'UTR (untranslated region) at residues 3008-3027 (NM_201414.2). This sequence exhibited characteristics of a parallel G-quadruplex structure as revealed by circular dichroism spectrophotometry. Further, as with other G-quadruplexes, the formation of this structure was dependent on the presence of potassium ions. This G-quadruplex has no apparent role in regulating transcription or mRNA stability as wild type and mutant constructs exhibited equivalent mRNA levels as determined by real time PCR. Instead, we demonstrate that this G-quadruplex negatively regulates APP protein expression using dual luciferase reporter and Western blot analysis. Taken together, our studies reveal post-transcriptional regulation by a 3'UTR G-quadruplex as a novel mechanism regulating APP expression.

Introduction

Amyloid plaques and neurofibrillary tangles are characteristic pathologic features of Alzheimer's disease (AD), a progressive neurodegenerative disorder and the most common form of dementia [129]. Amyloid plaques are formed from the amyloid β peptide (Aβ), which is a proteolytic product of the amyloid precursor protein (APP). APP is a type 1 transmembrane protein that is ubiquitously expressed in humans [130]. While the biological function of APP remains obscure, a large body of work indicates that APP plays a critical role in AD pathogenesis via production of Aβ [130]. APP undergoes regulated intramembrane proteolysis (RIP) by one of two proteases, α- or β-secretase. Cleavage by α-secretase in the non-amyloidogenic pathway releases a secreted APP fragment (s-APP α) as well as a transmembrane α C-Terminal Fragment (CTF). Cleavage by β-secretase in the amyloidogenic pathway produces s-APP β and β CTF (for review see [45, 131-134]). APP CTFs can be further cleaved by β-secretase to produce p3 and APP Intracellular domain (AICD) in the non-amyloidogenic pathway or Aβ and AICD in the amyloidogenic pathway [45, 134-136]. Aβ peptides can accumulate and form oligomers that eventually give rise to amyloid plaques [137]. The accumulation of Aβ oligomers can lead to synaptic loss and neurodegeneration [138]. Rare, early-onset forms of AD arise from mutations leading to elevated Aβ production. This change in Aβ can arise from heightened APP levels due to mutations in *APP* or from increased APP copy number as observed in Down's syndrome (Trisomy 21) [139, 140]. Early onset AD can also arise from elevated Aβ levels due to altered APP processing caused by mutations in the γ-secretase genes *PSEN1* or *PSEN2* [130].The accumulation of Aβ peptides is

thought to lead to tau hyperphosphorylation, which can result in synaptic dysfunction, neuronal death, and cognitive decline [64]. Elevated APP expression, and the associated increase in Aβ production via the amyloidogenic pathway, therefore has deleterious effects on both neuronal and cognitive function.

Decreased levels of APP also lead to pathological changes in the brain, as revealed by studies investigating genetically modified mice that lack APP. Acute knock down of APP in neuronal precursor cells prevents these cells from migrating into the cortical plate [55]. Additionally, mice lacking APP exhibit defects in synapse formation that manifest as decreased dendritic spine abundance [141]. The synapses that do form exhibit altered plasticity, as they have impaired long term potentiation [48, 141, 142]. Therefore, as with overexpression of APP, reduced levels of APP lead to negative changes in neuronal structure and function.

As both over- and under-expression of APP can be deleterious, identifying the endogenous mechanisms that normally maintain APP expression within the physiological range is of particular interest. Regulatory sequences within the 5' and 3' UTRs (untranslated regions) of an mRNA can affect its stability, transcription, and translation and therefore contribute to the spatial and temporal regulation of gene expression [143-147]. One mechanism whereby regulatory RNA sequences alter translation is through RNA secondary structures [148-150]. Guanine quadruplexes (G-quadruplexes), one such secondary structure [96, 115, 151], are DNA or RNA sequences containing repeating guanines arranged in a manner that facilitates intra-molecular assembly of stacks of guanine tetrads [114]. Stacking of these guanine tetrads is stabilized by monovalent cations, especially K^+ and Na^+ ions [151]. G-quadruplexes require two or more stacks of

guanine tetrads [152]. Both DNA and RNA G-quadruplexes form in cells [153, 154], although RNA G-quadruplexes are more stable than DNA G-quadruplexes [155].

Here we investigated the endogenous mechanisms that regulate APP expression. The APP 3'UTR contains a variety of regulatory sequences that affect the stability and ultimately translation of the APP mRNA [90]. We show that a G-rich region in the 3' UTR of *APP* is a G-quadruplex. Further, we demonstrate for the first time that this sequence negatively regulates APP gene expression in a post-transcriptional manner. These findings are consistent with previous reports demonstrating that 3'UTR G-quadruplexes can negatively regulate the expression of genes that harbor such structures. Moreover, our results suggest this secondary structure is a novel mechanism regulating APP gene expression and therefore may be an important factor contributing to AD pathogenesis.

Materials and Methods:

APP G-quadruplex identification and sequence conservation

Using Quadparser, the sequence for APP mRNA (NM_201414.2) was searched for putative G-quadruplex sequences following the sequence motif $(G_{\geq 2}N_{1-7})_3G_{\geq 2}$ which defines four repeats of at least two guanines (G) interrupted by stretches of one to seven nucleotides of any type (N) [156]. Alignment for APP 3'UTR G-quadruplex sequence was performed using QGRS-H Predictor software [157] using GeneBank Accession

numbers as indicated in the text for APP of different species. Potential G-quadruplexes were analyzed approximately 718 nucleotides downstream from the stop codon.

RNA preparation

RNA oligonucleotides (APP 3'UTR G-quad Wild type: 5'GGGGCGGGUGGGGAGGGG-3') and (APP 3'UTR G-quad Mutant: 5'-GGGGCGGGUGGGGAAAAA-3') were purchased from Dharmacon, Inc. and Integrated DNA Technologies. Li^+ ions prevent Na^+- or K^+-induced G-quadruplex formation. To ensure that G-quadruplexes did not form in the analyzed oligonucleotides prior to conducting experiments, the RNA was stored in buffers containing Li^+ ions. To replace cations in the RNA solution with Li^+, RNA was dialyzed as described previously [158] in an eight-well microdialysis apparatus (Gibco-BRL Life Technologies) at a flow rate of 25 mL/min. The RNA was initially dialyzed with 100 mM LiCl for 6 h to replace the RNA backbone cation, followed by nuclease-free water for another 6 h to remove excess LiCl, and finally with 10 mM LiCacodylate (pH 7.0) overnight. Concentrations of the dialyzed RNA were quantified by UV-spectroscopy, and the RNA was stored at –20^{o}C until the experiments.

Circular Dichroism (CD) Spectroscopy

CD spectroscopy experiments were conducted based on a previously published protocol [158] using a Jasco CD J810 Spectropolarimeter and analyzed with KaleidaGraph v.4.5.2 (Synergy Software). RNA oligonucleotides were prepared to a concentration of 2.5 μM

in 10 mM LiCacodylate (pH 7.0) by the dialysis procedure described above. Prior to CD, RNAs were denatured at 95 °C for 2 min and renatured at room temperature for 15 min for equilibration. Spectra were acquired every nanometer from 220-310 nm at 25°C. Each reported spectrum is an average of 2 scans with a response time of 2 s/nm. Data were normalized to concentration, oligonucleotide length and cuvette pathway to provide molar residue ellipticity values and smoothed over 5 nm [159].

Data fitting

CD-detected titrations were performed with KCl to determine the concentration of potassium ion (K^+) required to drive G-quadruplex formation. To determine $K^+_{1/2}$ values, ellipticity data as a function of K^+ concentration were fit with KaleidaGraph v. 4.5.2 (Synergy software) according to the apparent three-state Hill equation as previously described [158].

$$\varepsilon = \frac{\varepsilon_U + \varepsilon_I\left(\frac{[K^+]}{[K^+_{1/2}]_1}\right)^{n_1} + \varepsilon_F\left(\frac{[K^+]}{[K^+_{1/2}]_1}\right)^{n_1}\left(\frac{[K^+]}{[K^+_{1/2}]_2}\right)^{n_2}}{1 + \left(\frac{[K^+]}{[K^+_{1/2}]_1}\right)^{n_1} + \left(\frac{[K^+]}{[K^+_{1/2}]_1}\right)^{n_1}\left(\frac{[K^+]}{[K^+_{1/2}]_2}\right)^{n_2}} \qquad \text{(Equation 1)}$$

where ε is the normalized molar ellipticity, ε_U is the normalized CD signal for fully unfolded RNA, ε_I is the normalized CD signal for intermediate state RNA, and ε_F is the normalized CD signal for fully folded RNA. $[K^+_{1/2}]_1$ and n_1 are the $K^+_{1/2}$ and Hill coefficient values for the U-to-I transition, while $[K^+_{1/2}]_2$ and n_2 are the values for the I-to-F transition. Data were collected at the maximum wavelength (λ_{max}) in the spectrum.

Constructs and Site-Directed-Mutagenesis

The base vector for the luciferase experiments was pMIR-Report miRNA expression reporter vector which contains the firefly luciferase gene (Promega). To create the wild type (WT) construct, the entire 1.1 kb sequence of the APP 3'UTR (NM_201414.2) was placed 3' of the Luciferase gene stop codon. To create a 3'UTR mutant construct, site-directed mutagenesis (Stratagene, Quikchange mutagenesis kit) was carried out on the wild-type 3'UTR construct to mutate the final four guanine nucleotides of the G-quadruplex to adenine nucleotides (5'-CCCTGTTCATTGTAAGCACTTTTACGGGGCGGGTGGGGAAAAATGCTGGTCTTCAATTAC-3' and 5'-GTAATTGAAGACCAGCATTTTTCCCCACCCGCCCCGTAAAAGTGCTTACAATGAACAGGG-3') [38]. Mutagenesis was confirmed by DNA sequencing (Macrogen).

In order to create the human APP-APP 3'UTR over-expression plasmid, we amplified the coding region of human APP and sub cloned into the pmCherry-N1 vector using the NheI (5'-CGACGACGAGCTAGC ATGCTGCCCGGTTTGGCA-3') and XmaI sites (5'-TCGTCGTCGCCCGGGCGTTCTGCATCTGCTCAAAGAA-3'). The human wild-type 3'UTR of APP was amplified and sub cloned 3' of the APP stop codon using the AgeI site underlined (5'-CGACGAACCGGTACCCCCGCCACAGCAGC-3') sense and (5'-CGGCGGCGGACCGGTGCTCCTCCAAGAATGTATTTATTTAC-3') antisense. The resulting plasmid was then subjected to site-directed mutagenesis (QuikChange; Stratagene) to change the final four guanine nucleotides of the G-quadruplex to adenine nucleotides [160] . Mutagenesis was confirmed by DNA sequencing.

Using these APP overexpression constructs bearing either the wild type or mutant 3'UTR G-quadruplex, we inserted a c-Myc epitope tag before the APP_{695} stop codon using site-directed mutagenesis with mutagenic primers (5'gagcagatgcagaacgaacaaaaacttatttctgaagaagatctgtagcccgggatccac 3' and 5'gtggatcccgggctacagatcttcttcagaaataagtttttgttcgttctgcatctgctc 3'.) The underlined nucleotide sequence corresponding to the c-Myc amino acid sequence "EQKLISEEDL."

Cell Culture and Transfection

Experiments were carried out in HeLa and HEK293 cells (as indicated in the Results), purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum, L-glutamine (2%), penicillin (25,000 U/ml) and streptomycin (25,000 μg/ml). One day before transfection $4x10^5$ naïve HeLa or HEK293 cells were seeded in 6 well plates. The next day, the medium was replaced with a transfection medium containing the plasmids and Turbofect (ThermoScientific) according the manufacturer's instructions.

For the metabolic labeling experiments, HeLa cells were transfected using Lipofectamine 3000 (ThermoScientific) according to the manufacturer's recommendations. Briefly, the HeLa cells were cultured in complete Dulbecco's modified Eagles medium (DMEM) as described above. One day before transfection, HeLa cells were seeded in 10 cm tissue culture dishes such that they would reach 90% confluence the following day. On the next day, medium was aspirated and replaced with antibiotic free medium containing 10% FBS and 2%L-glutamine. APP-Myc constructs containing either wild type or mutant

3'UTR G-quadruplex sequence were transfected. 6 hours post transfection, transfection medium was aspirated and replaced with complete medium.

Metabolic Labeling using *L*-azidohomoalaine (AHA)

24 hours post transfection, cells in 10 cm dishes were prepared for metabolic labeling to examine newly synthesized proteins. Cells were washed twice with warm PBS. DMEM containing only high glucose was added to cells to deplete endogenous methionine and cysteine for 45 minutes. Next, the medium was replaced with DMEM containing 10% dialyzed FBS, 2% L-glutamine, .2 mM L-cysteine, 25 mM HEPES, 1 mM sodium pyruvate, penicillin (25,000 U/ml) and streptomycin (25,000 μg/ml), and 4 mM *L*-azidohomoalanine (Click Chemistry Tools) for 4 hours. After this incubation period, cell lysates were collect in RIPA buffer and prepared for immunoprecipitation.

Immunoprecipitation

Following metabolic labeling of cells, lysates were prepared for immunoprecipitation using Dynabeads magnetic Protein G Beads (Life Technologies) following the manufacturer's instructions. The antibody used for immunoprecipitation was 9B11 C-Myc antibody (Cell Signal) to capture APP_{695}Myc (wild type or mutant G-quadruplex) reporter constructs but not the endogenous APP. Following elution, 40% of the eluate along with the immunoprecipitation input and flow through were used for Western Blot Analysis to detect APP using the c1/6.1 mouse monoclonal antibody. The remainder of the eluate was used for the "Click Chemistry" reaction.

Click Chemistry Reaction

To perform this reaction, IP eluate was subjected to the Click Chemistry Protein Reaction Kit (Click Chemistry Tools). In this reaction a desthiobiotin molecule containing an alkyne group forms a covalent bond to proteins containing the AHA labels (via the AHA's azide group). This reaction followed the manufacturer's instructions with the exception that the 30 min incubation time was changed to 1 hour. Following the reaction, samples were prepared for Western blot.

Western Blot

24 hours post transfection, cells were lysed in RIPA cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing 1X protease inhibitor cocktail (Thermo Scientific). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. The resulting supernatants were transferred to a new microcentrifuge tube and the cell lysate protein concentration was determined using the BCA protein assay kit (Pierce) according to the manufacturer's instructions.

Equal quantities of protein (~40 μg) were mixed with loading buffer and loaded into the wells of 4–12% Bis-Tris polyacrylamide gels (Invitrogen) along with molecular weight standard (LiCor). Gels were run using MES running buffer and transferred to PVDF membrane (Immobilon PSQ, Millipore) using a semi-dry transfer apparatus (Owl Scientific) and NuPage transfer buffer (Invitrogen). After transfer, membranes were blocked with Odyssey Blocking Buffer (LiCor) for 1.5 hours. Next, the blocking buffer was removed and the membrane probed overnight at 4°C with blocking buffer containing C1/6.1 C-Terminal antibody (a gift from Paul Matthews;, 1:4000) and an antibody to β-

actin (Sigma; 1:10,000) which were diluted in blocking buffer. Membranes were washed with 1X PBST for 5 minutes (4 times). After the washes, the membrane was probed for 1 hour at room temperature with goat anti-mouse 2° Antibody (800 nm; LiCor) at 1:10,000 diluted in blocking buffer. For the metabolic labeling studies, IRdye 800-conjugated Streptavidin (1:10,000; Licor) was used to detect proteins containing desthiobiotin. Membranes were washed extensively and then scanned using the LiCor Odyssey system. Band intensities were quantified using the Odyssey software.

Luciferase Assays

Cells were split the day prior to transfection and plated to 40-50% confluency in either 24- or 96-well plates. On the day of transfection, media was aspirated and replaced with fresh media and plasmids transfected using Turbofect (ThermoScientific) according to the manufacturer's instructions. An excess of pMIR-Luciferase plasmid was transfected relative to the transfection control plasmid (pRL-TK Renilla luciferase); a 40 to 1 molar ratio was utilized. 24 hours post-transfection, media was removed and Glo Lysis Buffer (Promega) was added to each well to lyse cells. These lysates were frozen (-80° C) prior to performing Dual Glo Luciferase Assay (Promega). Firefly Luciferase and pRL-TK Renilla luciferase activity were measured per the manufacturer's instructions. In all cases, firefly luciferase values were normalized to Renilla luciferase values.

RNA isolation and Real Time PCR

To collect the lysates for RNA isolation, media from the 6-well plates was aspirated and replaced with 600 µL of RLP buffer (Qiagen, RNeasy Mini Kit) and 60 µL of β-mercaptoethanol (Sigma). All equipment used in the RNA isolation procedure was

cleaned with RNaseZap (Ambion) solution. Total RNA was DNase treated by RQ1 RNase-free DNase (Promega), and the RNA concentration was determined spectrometrically (Nano-Drop; Thermo Scientific). cDNA synthesis from total RNA utilized random primers (Invitrogen), Super Script II (Invitrogen), RNase H (Invitrogen), dNTPs (Invitrogen), and RQ1 (Promega). Synthesized cDNA was utilized for real time PCR. We designed primers and probes for firefly and renilla luciferase (Firefly Luciferase primers- 5'-GCTATTCTGATTACACCCGAGG-3', 5'-TCCTCTGACACATAATTCGCC-3', 5'-6-FAM-TCCAGATCCACAACCTTCGCTTCAAA-TAMRA-3'; Renilla Luciferase primers- 5'-CAAAGAGAAAGGTGAAGTTCGTC-3', 5'-GTGGTAAACCTGACGTTGTAC-3', 5'-FAM-ATCATGGCCTCGTGAAATCCCGT-TAMRA-3') and used these in combination with a BioRad 384 well real time thermocycler (CFX384). Taqman Universal PCR Master Mix was used following the manufacturer's cycling conditions.

Aβ ELISA

$A\beta_{40}$ ELISA (Wako) was performed using conditioned media from cells transfected with APP_{695} overexpression plasmids containing either the wild type or mutant G-quadruplex sequence following the manufacturer's instructions.

Statistical Analysis

All experiments were repeated 3 times unless stated otherwise. All errors are shown as standard error of the mean. Equal variance was assumed for the two-sample student's t-test (95% confidence interval). Q test was used to reject outliers at the 95% confidence interval. * indicates a $P < 0.05$, ** indicates a $P < 0.01$, and *** indicates a $P < 0.001$.

Results

Bioinformatic identification and sequence conservation of an APP 3'UTR G-quadruplex

To investigate the post-transcriptional regulation of APP expression, we asked whether the human *APP* mRNA contains a G-quadruplex. Using Quadparser [156], we searched the APP mRNA sequence (NM_201414.2) for putative G-quadruplex sequences following the sequence motif, $(G_{\geq 2}N_{1-7})_3G_{\geq 2}$ which defines four repeats of at least two guanines (G) interrupted by stretches of one to seven nucleotides of any type (N). This approach predicted two putative G-quadruplexes within APP mRNA (**Figure 1.1A**). One such sequence is located within the protein coding region beginning at nucleotide 957, consistent with earlier findings [96]. This potential G-quadruplex is predicted to be relatively weak since it has the potential to form a quadruplex with only two stacks of guanine tetrads and the intervening loops are relatively long with 4 nucleotides each [161]. A second putative G-quadruplex was identified within the 3'UTR beginning at nucleotide 3008. This 3'UTR sequence was recently identified independently by a bioinformatic analysis but was not experimentally investigated [162]. This putative G-quadruplex could form a relatively strong G-quadruplex since it has the potential to form a quadruplex comprised of three guanine tetrads and the intervening loops are only 2 or 1 nucleotides in length [152]. Since the G-quadruplex found in APP 3'UTR is approximately 718 nucleotides from the stop codon, we searched the APP gene of several species for potential G-quadruplexes around the same nucleotide distance from the stop codon and was able to obtain an alignment for the comparison of the genes using GQRS-H Predictor software [157] (**Figure 1.1B**). The potential functional importance of this 3'UTR G-quadruplex is highlighted by its conservation in *APP* genes in various species

(**Figure 1B**). These findings suggest the presence of functional G-quadruplexes in the *APP* mRNA.

Structural Confirmation of the APP 3'UTR Putative G-quadruplex

To validate that the sequence identified by the bioinformatic approach is a bona fide G-quadruplex, we performed a structural characterization of this sequence. Several factors contribute to the folding of an RNA into a G-quadruplex, including the sequence itself (guanine tracts, loop sequence, and loop length) as well as the cellular environment (pH, temperature, and the concentration and identities of monovalent cations) [152, 163-165]. Importantly, potassium (K^+) ions preferentially stabilizes G-quadruplex structures in comparison to sodium (Na^+) and lithium (Li^+) ions [166]. G-quadruplex formation can be monitored through key spectral signatures using circular dichroism (CD) spectroscopy. To test whether the 3'UTR sequence forms a G-quadruplex, we used an RNA oligonucleotide bearing the putative APP 3'UTR G-quadruplex and performed K^+ ion titration monitored by CD. The first immediate observation is that CD spectra of this RNA includes a negative peak at 240 nm and a positive peak at 262 nm (**Figure 2.2A**), which are the distinctive CD signatures for a parallel G-quadruplex structure [158, 167]. We next investigated the K^+ ion dependence of this G-quadruplex structure. Plotting the change in ellipticity versus K^+ ion concentration revealed a three-state transition (**Figure 2.2B**), with a $K^+_{1/2}$ of ~3 μM and ~18 mM. As the physiological K^+ concentration is ~150 mM , this result suggests that the APP 3'UTR G-quadruplex is fully folded *in vivo*, with a maximum of 3-quartet planes. We then used CD to compare the oligonucleotide representing the wild-type APP 3'UTR G-quadruplex to the spectrum of the oligonucleotide representing a APP 3'UTR G-quadruplex sequence in which the fourth

set of G repeats was replaced with adenines (APP 3'UTR G-Quad Mutant) [160, 168]. This was conducted in the presence of 150 mM KCl, which induces G-quadruplex formation in the wild-type sequence. In this mutated oligonucleotide we observed a significant decrease in the 262 nm peak, indicating a decrease in population of the G-quadruplex fold (**Figure 2.2C**). Taken together, our CD data support the presence of a parallel G-quadruplex that is stabilized by K^+ ions.

The APP 3'UTR G-quadruplex does not regulate expression levels of the APP RNA

We next investigated the functional significance of this G-quadruplex. As these structures can play roles in transcription, RNA stability and translation[151], we wanted to investigate what role the APP 3'UTR G-quadruplex has on gene expression. We began these studies by investigating whether the G-quadruplex regulated RNA levels, which would suggest a role in either transcription and/or RNA stability. To perform these studies, we utilized a luciferase reporter construct in which the human APP 3'UTR was inserted after the stop codon of the firefly luciferase gene [106]. To gain insight into the role of the G-quadruplex in regulating expression we created a parallel luciferase construct in which we disrupted the 3'UTR G-quadruplex structure by changing the fourth set of guanine repeats to adenines thereby disrupting tetrad formation [160, 168]; this mutant is analogous to the mutant oligonucleotide sequence used in the CD studies above. To test whether the G-quadruplex affected the expression levels of these transcripts, we transfected HEK293 cells with either construct using identical transfection conditions. Using qPCR to measure luciferase mRNA levels in these cell populations, we could detect no differences between luciferase mRNA having either the wild type or mutant G-quadruplex (**Figure 2.3A**). The APP 3'UTR G-quadruplex therefore does not

play any substantial role in regulating the expression at the level of transcription or RNA stability.

The APP 3'UTR G-quadruplex Negatively Regulates APP Protein Levels

We then investigated if the APP 3'UTR G-quadruplex plays a role in translation. The wild type and mutant luciferase reporter constructs were individually transfected into HEK293 cells. 24 hours post-transfection, we measured the luciferase activity as a proxy for luciferase protein expression levels from the two constructs. Disruption of the G-quadruplex structure significantly increased luciferase activity by 80% (**Figure 2.3B**) compared to the intact, wild type, G-quadruplex. These findings were consistent with results obtained in parallel experiments using HeLa cells (data not shown) indicating that they are independent of the cell type. These findings suggest that translation is more efficient from the construct containing the mutant sequence than that containing the wild type G-quadruplex.

We next sought to establish a system in which we could measure the effects of this G-quadruplex on APP expression. For these experiments, we used a plasmid encoding the 695 amino acid isoform of human APP (APP_{695}) followed by either the wild-type- or G-quadruplex-mutated human APP 3'UTR. These constructs were transiently transfected separately into HeLa cells, which do not express the 695 amino acid isoform of APP [169, 170]. Cell lysates were collected 24 hours post-transfection and subjected to Western blot analysis. Consistent with previous findings [169, 170], we could not detect APP_{695} in untransfected cells (**Figure 4A**). In contrast, transfected cells expressed readily detectable APP_{695} (**Figure 2.4A**). Therefore, we can specifically detect exogenous full-length APP_{695} using Western blot analysis.

We then used this system to compare APP expression from cells expressing APP_{695} followed by either the wild type or G-quadruplex-mutated 3'UTR. Mutating the G-quadruplex resulted in an approximate two-fold increase in steady state levels of full-length APP_{695} (**Figures 2.4A and 2.4B**), consistent with the results obtained using the luciferase assay. To gain further insight into APP processing, we measured the abundance of the CTF that was produced from APP_{695} having either the wild type or mutant 3'UTR G-quadruplex. We measured the level of endogenous APP CTF in mock transfected cells. We then measured the levels of exogenous and endogenous APP CTF in cells transfected with the APP695 3'UTR WT G-quad and APP695 3'UTR Mutant G-quad constructs individually. We subtracted the intensity of the APP CTF band of mock transfected cells from that of cells expressing either of the APP constructs to obtain the effect of the 3'UTRs on exogenous APP CTF levels (**Figure 2.4C**). This analysis revealed a significant increase in the amount of exogenous APP CTF in the mutant compared to the wild type control. We next investigated whether the increased APP expression led to increased production of Aβ peptides. As expected, ELISA quantification of endogenous and exogenous Aβ indicated that there was a 1.6-fold increase in Aβ levels from cells transfected with APP containing the mutant G-quadruplex sequence compared to the wild type G-quadruplex (**Figure 2.4D**). The effects we observe on APP proteolytic cleavage are due to increased APP expression that results from mutating the 3'UTR G-quadruplex. Taken together, these data indicate that the 3'UTR G-quadruplex negatively regulates APP levels.

Translational Control of APP by G-quadruplex

Loss of the G-quadruplex leads to an increase in APP protein levels (**Figure 2.4**) without affecting the *APP* transcript levels (**Figure 2.3A**), suggesting a role for this structure in translational control. To test this prediction, we labeled newly synthesized proteins during a discrete window and asked whether more APP protein was produced from the construct in which the G-quadruplex was mutated. For these experiments, we used HeLa cells that expressed myc-tagged APP constructs followed by either the wild type or G-quadruplex-mutated 3' UTR (**Figure 2.5A**). These cells were metabolically labeled for four hours with L-azidohomoalanine (AHA), a methionine analog that is incorporated into newly synthesized proteins during translation and can be subsequently detected using click chemistry-based approaches. In this approach a desthiobiotin molecule containing an alkyne group forms a covalent bond to the AHA molecule incorporated into newly synthesized proteins (**Figure 2.5B**) [171]. Specifically, we immunoprecipitated the exogenous APP using antibodies that recognize myc, biotinylated the AHA-containing APP, and used immunoblotting to determine the extent to which this immunoprecipitated APP had been synthesized during the labeling window (**Figure 2.5C**). Using this approach, we determined that the mutant construct resulted in an increase in newly synthesized APP of 19.4% ± 5.3% (mean ± SEM; ratio paired t-test $p = 0.0052$; $n=6$) during the labeling window (**Figure 2.5D**). Taken together with the previous findings, these results demonstrate that the 3'UTR G-quadruplex modulates APP protein expression by negatively regulating translation.

Discussion

In this study, we have analyzed the human APP mRNA sequence for potential regulatory elements and identified a previously uncharacterized G-quadruplex within its 3'UTR. The formation of the intact RNA G-quadruplex was confirmed by CD spectroscopy. Importantly, this structure is stable at physiologic K^+ concentrations. Using two independent expression constructs, our results demonstrate that this G-quadruplex negatively regulates gene expression in a post-transcriptional manner. In addition to the negative regulation of APP via the 3'UTR G-quadruplex, we further showed that APP overexpression resulting from loss of regulation by the G-quadruplex led to increased Aβ levels. Further studies are needed to elucidate the mechanism by which the APP 3'UTR G-quadruplex regulates APP gene expression.

G-quadruplex-forming sequences can be found throughout mRNAs, including within the 5'UTR, coding regions, and 3'UTR. G-quadruplexes in the 5'UTR have the potential to suppress mRNA translation by blocking initiation factors binding [172] that are required for activating cap dependent translation initiation [173]. G-quadruplexes in the coding regions may have a role in stalling translational elongation [174, 175]. G-quadruplexes in the 3'UTR can be involved in translational repression [176], polyadenylation-dependent mRNA stability [177], and dendritic mRNA targeting [162]. Our data show that the G-quadruplex found in the APP 3'UTR regulates APP gene expression at the translational level.

G-quadruplexes have been found in two other genes whose proteins play crucial roles in APP proteolysis and AD etiology – ADAM10 and BACE1. The α-secretase ADAM10 contains a 5'UTR G-quadruplex that represses translation [178]. BACE1, the β-secretase gene, contains an exonic G-quadruplex that drives BACE alternative splicing. Formation of this G-quadruplex produces a shorter, inactive BACE isoform, while disruption of this G-quadruplex results in the full-length, active BACE isoform which leads to increased Aβ production [179]. The presence of the G-quadruplex in these mRNAs may lead to coordinated regulation and cleavage of APP in response to specific cellular conditions.

Previous work has demonstrated that G-quadruplexes may act as negative regulators of gene expression [180-184] and can exert their regulatory effects by interacting with RNA binding proteins that repress translation [185, 186]. Over three dozen proteins have been reported to bind the APP mRNA [90, 162, 187, 188]. Several of these proteins – including Nucleolin, hnRNP A, Fus, and FMRP– bind to G-quadruplex sequences[186, 189-193]. Whether these proteins interact with the G-quadruplex in the 3'UTR of APP is not known. Moreover, such proteins may interact with the G-quadruplex in concert with other factors; for instance, it has been shown for other mRNAs that RNA binding proteins that interact with G-quadruplexes can require additional protein co-factors to facilitate translational regulation [194, 195] . This remains an important area for future study. Additionally, recent studies have demonstrated that G-quadruplexes may interact with other translational regulators such as microRNAs [195, 196]. Overall, our results show that dysregulated APP translation due to disruption of the G-quadruplex in the 3'UTR lead to elevated levels of APP. Since increased APP levels can lead to AD, it will be of interest to determine whether AD cases arise from dysregulated APP expression

due to mutations that disrupt the APP 3'UTR G-quadruplex and/or in the RNA binding proteins that interact with this sequence.

Acknowledgements

We would like to thank members of the Saunders lab, Dr. Jeffrey Twiss for constructive comments on this work. We would also like to thank Dr. Paul Matthews for sharing the C1/6.1 antibody.

Figure Legends

Figure 2.1. APP contains a putative G-quadruplex. (A) Schematic of the APP mRNA that contains the putative G-quadruplex sequence in the coding region at position 957 (as identified by Westmark *et al*. 2007) and the G-quadruplex sequence in the 3'UTR (discussed in this paper) at position 3008. (B) G-quadruplex consensus sequence and comparison of the G-quadruplex sequence in the 3'UTR of human APP with the 3'UTR APP for other species.

Figure 2.2. CD potassium ion titration of the *APP* 3'UTR G-quadruplex shows that it forms *in vitro* and is in parallel topology with 3-state folding. (A) CD spectra collected as a function of K^+ ion concentration. K^+-mediated G-quadruplex folding is performed at 2.5 µM RNA under 10 mM lithium cacodylate (LiCac) (pH 7.0), with K^+ ion concentration ranged from 0 to 1 M. The positive peak at ~260 nm and negative peak at ~240 nm are CD signatures for parallel topology of G-quadruplex. (B) CD signal (ellipticity monitored at 262 nm) as a function of K^+ ion concentration from panel A shows clear three-state transitions in G-quadruplex folding. The fitting was performed using equation 1 (see Material and Methods). At physiological K^+ ion concentration (~150 mM), the G-quadruplex is fully folded. The $K^+_{1/2}$ and Hill coefficients (n) are provided in the plot. (C) CD titration and comparison of APP 3'UTR wild-type and mutant G-quadruplex sequence. 2.5 µM RNAs were used under 10 mM LiCac (pH 7.0) and physiological 150 mM or 0 mM K^+ ion concentration The GGGG to AAAA substitution in the mutant disfavor G-quadruplex formation as evident by the reduction in CD characteristic signals for G-quadruplex (compare blue and red), and yield similar CD signal to the wild type sequence at 0 mM K^+ ion concentration (green).

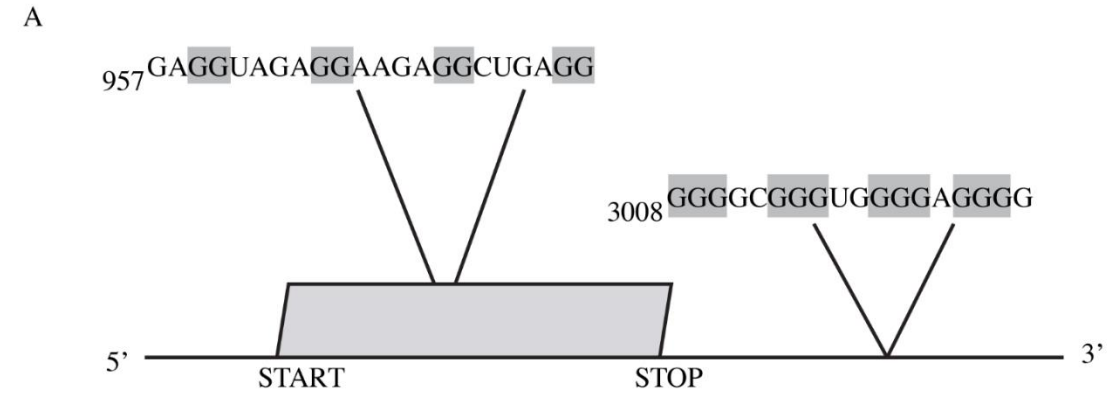
Figure 2.3. G-quadruplex regulation of Luciferase gene expression. (A) mRNA levels as assessed by qPCR and represented $\Delta\Delta C_T$ values represents the normalization of the ΔC_T of firefly luciferase to the ΔC_T renilla luciferase then normalized to the wild type. (B) Quantification of Dual Luciferase Assay comparing the wild type G-quadruplex sequence to the mutant G-quadruplex sequence (G-quad Mut). Empty Firefly Luciferase plasmid was used as a control which does not contain the 3'UTR of APP. Firefly Luciferase values were normalized to Renilla Luciferase.

Figure 2.4. G-quadruplex regulation of APP gene expression. (A) Western blot analysis of cells transfected with reporter constructs containing APP $_{695}$ coding sequence with wild type (G-quad WT) or mutant (G-quad Mut) sequence. Mock Transfection was used to confirm that these constructs were over-expressed in HeLa cells. Antibody C1/6.1 recognizes both full-length APP_{695} and CTF. β-Actin was used as a loading control. (B-C) Quantification of Western blots as in 4A with (B) APP levels normalized to β-Actin and (C) Endogenous CTF intensity values were subtracted from total CTF intensity values to obtain exogenous CTF values which were then normalized to β-Actin values. (D) Aβ ELISA quantification of total Aβ levels from conditioned medium of cells transfected with APP constructs containing the wild type or mutant 3'UTR G-quadruplex.

Figure 2.5. G-quadruplex regulation of APP translation. (A) Western blot analysis of cells transfected with reporter constructs containing APP $_{695}$ coding sequence containing a C-terminal myc tag with either wild type (WT G-quad) or mutant (Mut G-quad) G-quadruplex sequence. Mock transfection was used to confirm that these constructs were over-expressed. Antibody 9B11 (anti-Myc) was used to detect APP-Myc. (B) Schematic

representation of methods taken to identify newly synthesized APP (See methods). (C) Western blot analysis for immunoprecipitation demonstrating the successful pull down of Myc-tagged APP constructs (IP: using 9B11 anti myc mAb, IB: C1/6.1 anti APP). (D) Western blot analysis of total APP (top panel, IB: C1/6.1 anti APP) and newly synthesized APP (bottom panel, IB: Streptavidin). Statistical analysis was performed by normalizing the newly synthesized APP/Total APP (Streptavidin/C1/6.1) using a ratio paired t-test using Prism 6.0g for Mac.

Figure 2.1



B

H. sapiens	GGGGCGGGUGGGGAGGGG	Nucleotides 3008-3025 NM_201414.2
M. musculus	GGGU--GGG----GGGAGGGG	Nucleotides 3147-3131 NM_001198826.1
C. familiaris	GGGUAGGGGUGGGGGGGA	Nucleotides 3041-3061 NM_001006601.2
G. gorilla	GGGC--GGGUGGGGAGGGG	Nucleotides 2837-2854 XM_004062642.1
P. troglodytes	GGGC--GGGUGGGGAGGGG	Nucleotides 3051-3068 XM_002803170.1
O. aries	GGGUUGGGGAGGGUGGGG	Nucleotides 3136-3156 XM_004003805.1

Figure 2.2

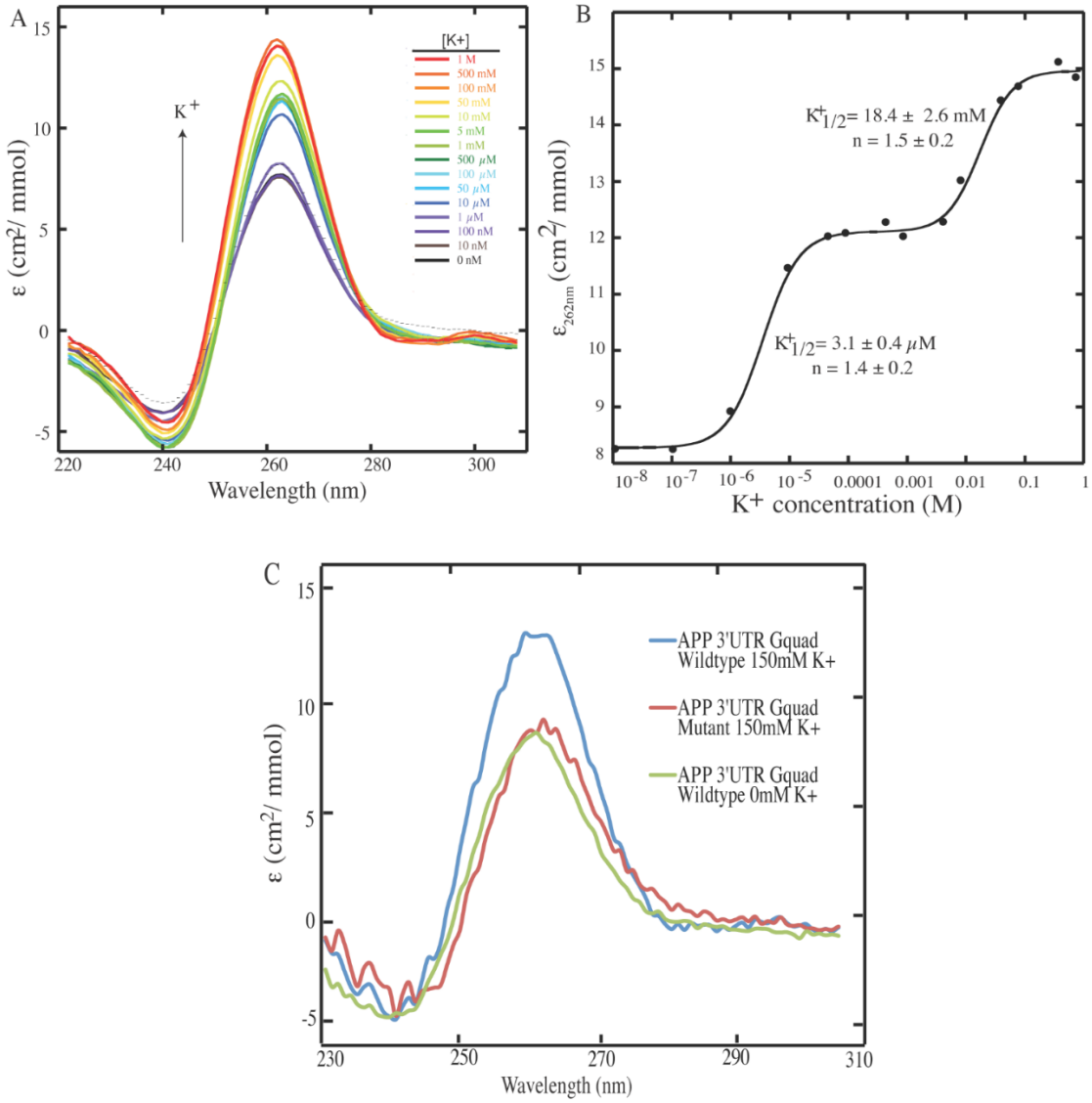

A
ε (cm²/ mmol)
Wavelength (nm)
K+
[K+]
1 M
500 mM
100 mM
50 mM
10 mM
5 mM
1 mM
500 μM
100 μM
50 μM
10 μM
1 μM
100 nM
10 nM
0 nM
B
ε262nm (cm²/ mmol)
K+ concentration (M)
K+1/2= 18.4 ± 2.6 mM
n = 1.5 ± 0.2
K+1/2= 3.1 ± 0.4 μM
n = 1.4 ± 0.2
C
ε (cm²/ mmol)
Wavelength (nm)
APP 3'UTR Gquad Wildtype 150mM K+
APP 3'UTR Gquad Mutant 150mM K+
APP 3'UTR Gquad Wildtype 0mM K+

Figure 2.3

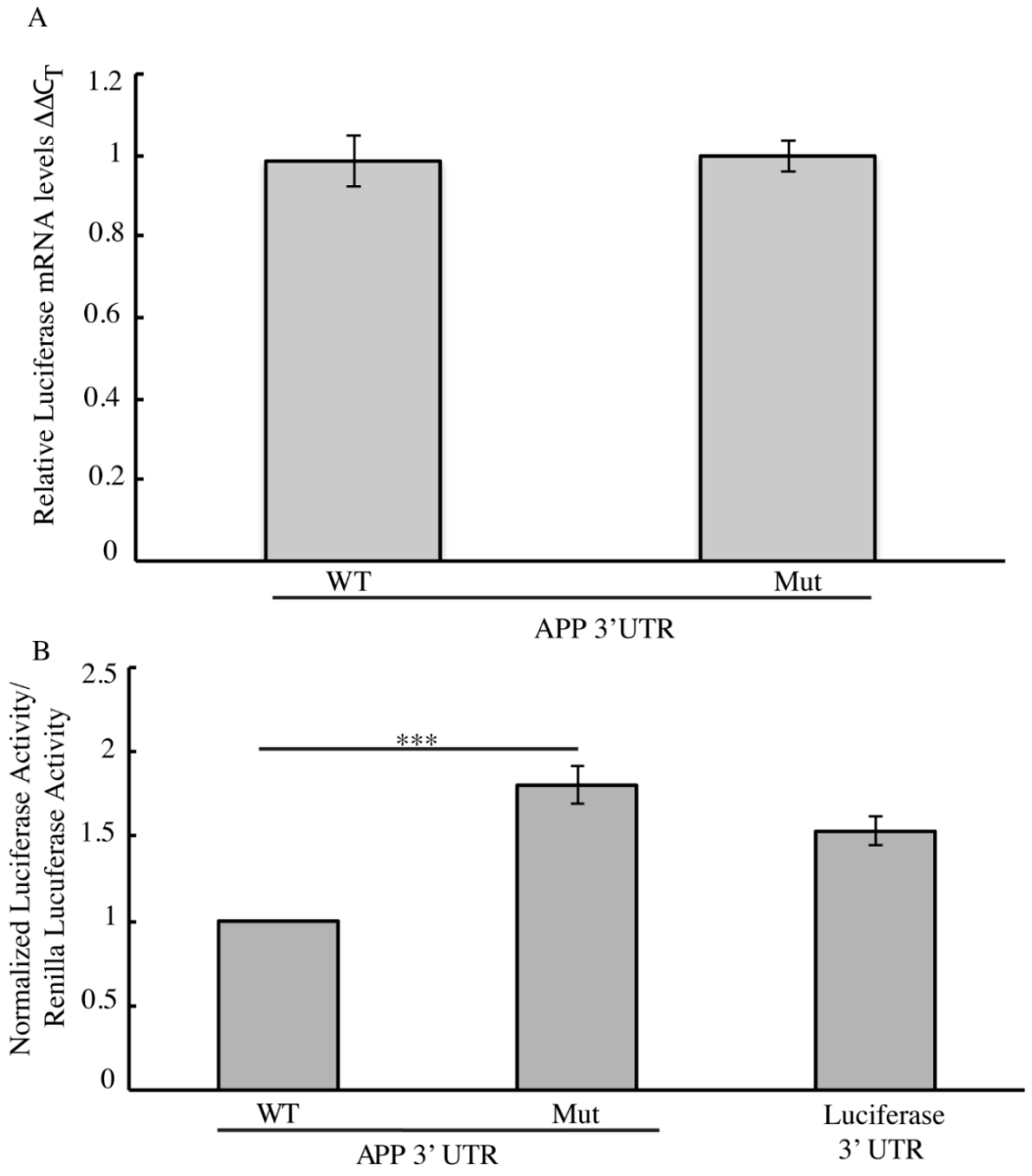

A
Relative Luciferase mRNA levels ΔΔC_T
1.2
1
0.8
0.6
0.4
0.2
0
WT
Mut
APP 3'UTR
B
Normalized Luciferase Activity/
Renilla Lucuferase Activity
2.5
2
1.5
1
0.5
0
***
WT
Mut
APP 3' UTR
Luciferase
3' UTR

Figure 2.4

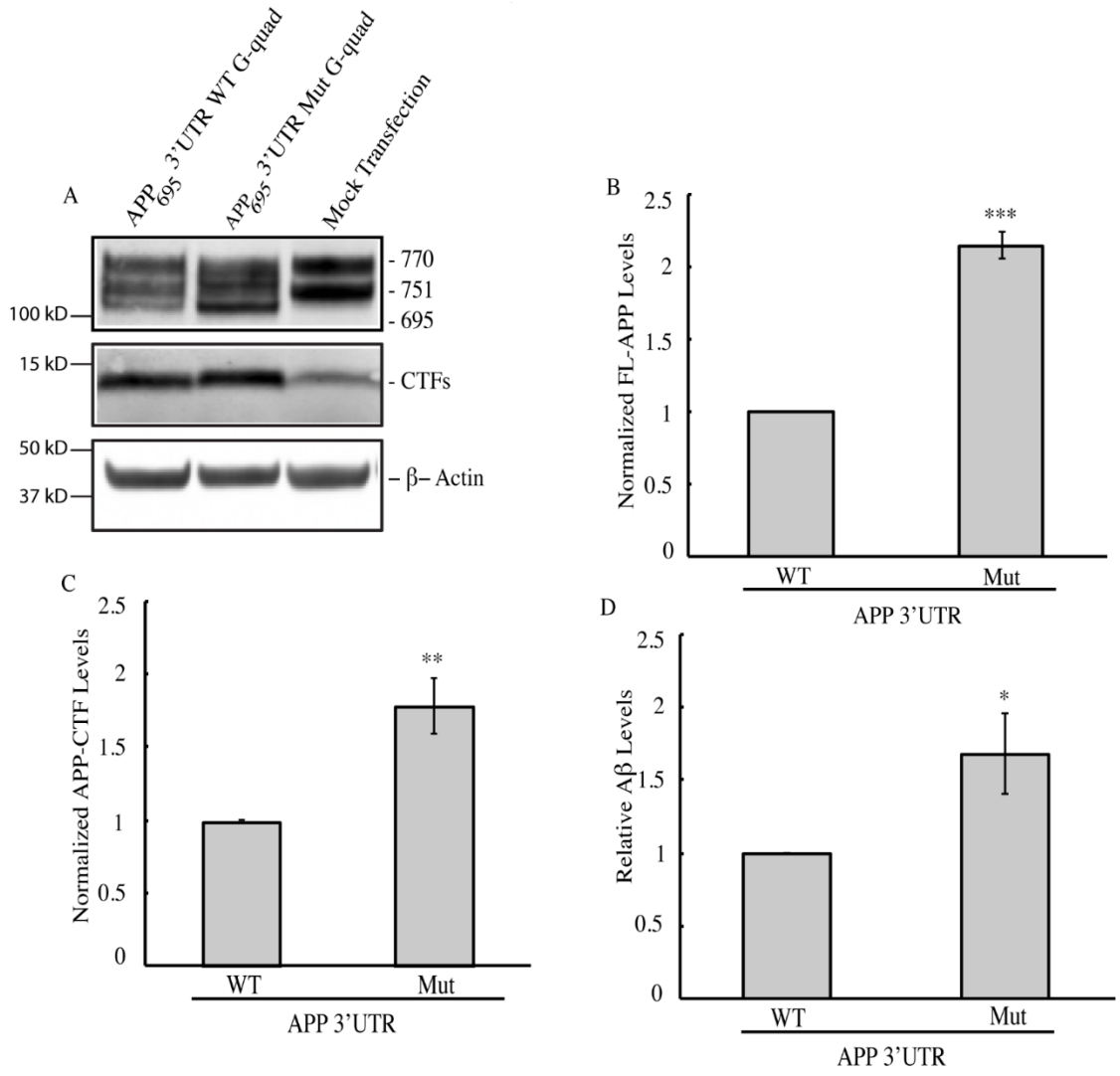
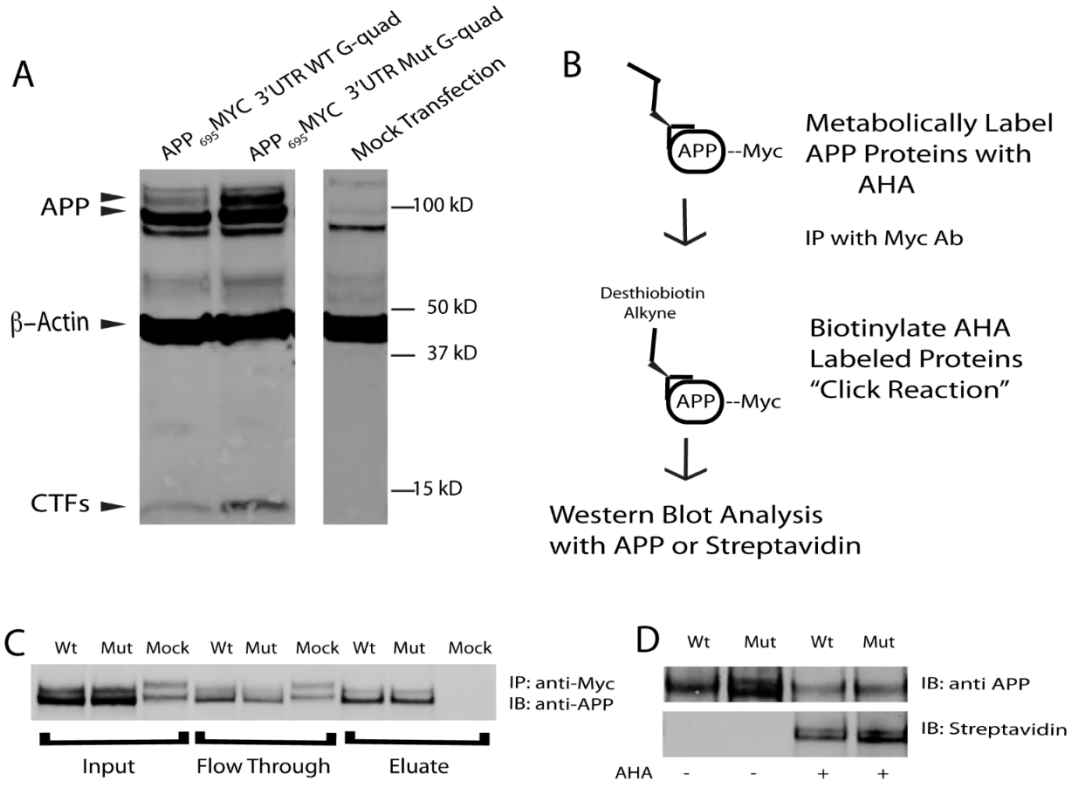

A
APP695 3'UTR WT G-quad
APP695 3'UTR Mut G-quad
Mock Transfection
100 kD
15 kD
50 kD
37 kD
- 770
- 751
- 695
- CTFs
- β- Actin
B
Normalized FL-APP Levels
***
WT
Mut
APP 3'UTR
C
Normalized APP-CTF Levels
**
WT
Mut
APP 3'UTR
D
Relative Aβ Levels
*
WT
Mut
APP 3'UTR

Figure 2.5


A
APP 695 MYC 3'UTR WT G-quad
APP 695 MYC 3'UTR Mut G-quad
Mock Transfection
APP
β–Actin
CTFs
100 kD
50 kD
37 kD
15 kD
B
APP --Myc
Metabolically Label APP Proteins with AHA
IP with Myc Ab
Desthiobiotin Alkyne
APP --Myc
Biotinylate AHA Labeled Proteins "Click Reaction"
Western Blot Analysis with APP or Streptavidin
C
Wt Mut Mock Wt Mut Mock Wt Mut Mock
IP: anti-Myc
IB: anti-APP
Input
Flow Through
Eluate
D
Wt Mut Wt Mut
IB: anti APP
IB: Streptavidin
AHA - - + +

Chapter 3

Secrets of the 3'UTR: Translational Regulation of the Amyloid Precursor Protein

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Abstract

Amyloid Precursor Protein (APP) plays a pivotal role in Alzheimer's disease. Increased levels of APP correlate with an increase in amyloid β (Aβ), which is the cleavage product of APP. Cleavage of APP by β secretase and sequentially by γ secretase releases Aβ, which can then form aggregates leading to the accumulation of plaques seen in Alzheimer's disease. While the functional role of APP is not well understood, research has shown that increased levels of the amyloid precursor protein lead to the increase in amyloid-β production. Therefore, dysregulation of APP translation contributes to Alzheimer's Disease. Translation is often regulated by cis regulatory elements in the 5' and 3' untranslated regions of the mRNA and by trans acting factors that recognize the sequences found therein. These regulatory elements primarily target the initiation step of translation, which is the rate limiting step in translation. Post-transcriptional regulation of

gene expression serves as a very important checkpoint between transcription and translation of the mRNA since the regulatory elements can influence mRNA stability, localization, and translation rate. Therefore, it is important that normal levels of APP be maintained which is necessary for its function. Research from our lab and others has demonstrated that APP translation can be influenced by cis and trans regulatory elements. Here we focus on the APP 3' untranslated region and how the regulatory elements found therein impact APP protein levels.

Introduction

The amyloid precursor protein (APP) plays a central role in the Alzheimer's disease (AD) pathogenesis. APP is type 1 transmembrane that contains a large N-terminal domain which faces extracellularly as well as a smaller intracellular C-terminal tail [43]. APP can be alternatively spliced to give rise to eight different isoforms with 695, 751, and 770 being the predominant isoforms. In the brain, APP is localized to presynaptic terminal axons as well as in dendrites [6, 42]. The biological function of APP is not well understood; however, APP can modulate neurite outgrowth and neuronal migration and has also been shown to play a role in synapse formation and maintenance [46]. APP can be proteolytically cleaved by secretases in one of two pathways referred to as the amyloidogenic pathway; which leads to the generation of amyloid beta(Aβ) and the non-amyloidogenic pathway [58]. In the amyloidogenic pathway, APP is cleaved by β secretase releasing and subsequently cleaved by γ secretase to release the Aβ peptide. In the non-amyloidogenic pathway, APP is cleaved by α and γ secretases; however, α secretase cleavage occurs with the Aβ sequence and thus prevents Aβ from forming [58]. Therefore, APP and Aβ are plays a pivotal the pathogenesis of (AD).

In additional to proteolytic cleavage of APP to generate Aβ, over-expression of APP protein levels leads to an increase in the production of Aβ. APP is located on chromosome 21 and there has been research indicating that individuals with trisomy 21 (Down Syndrome (DS)) are at a high risk of developing AD [9, 197, 198]. Several studies suggest there is a dose-dependent increase of APP in individuals with DS, as those individuals have an increase in APP levels which corresponds to an increase in Aβ levels [199]. Interestingly, individuals with DS develop an early onset of AD starting with evidence of neuropathological hall marks of AD, Aβ plaques and neurofibrillary tangles, around age 40 [9, 200]. In addition to over expression of APP due to trisomy 21, the Swedish mutation found in APP causes an increase in APP levels and increases the cleavage of APP by beta and gamma secretases, thereby releasing more Aβ [201, 202].

Since APP protein levels can influence Aβ generation, it is important to understand how APP expression is regulated. APP expression can be regulated transcriptionally, post-transcriptionally, and post-translationally [90]. Post-transcriptional regulation is an important intermediate between transcription and translation due the fact that several regulatory elements, such as RNA binding proteins or microRNAs, [203]. These regulatory elements are found throughout the APP mRNA; however, this review will focus on post-transcriptional regulation of APP from regulatory elements in the 3' untranslated region (UTR) and it they regulate the translation APP.

Overview of Translation

Maintenance of cellular protein levels requires an orchestrated series of processes which includes mRNA processing, degradation, stability, localization, and translation [204].

Previously, it thought that mRNA levels could be an indicator of protein abundance [205]; however, research has shown that mRNA levels poorly correlate with protein levels. Advances in technology has given insight into these regulatory processes that occur after an mRNA transcribed, and how they contribute to maintaining protein abundance [204]. Once an mRNA is transcribed, it is exported from the nucleus and serves as a substrate for translational control [206]. mRNAs are capped at the 5' end and contains a poly-A tail at the 3' end which promote the circularization of the mRNA and recruit translation initiator proteins, which in turn promotes translation [207, 208].

Control of translation can be regulated by targeting global translation or by targeting the specific mRNA [206]. Translation consists of initiation, elongation, and termination. The primary target in translational control is the initiation step. In translation initiation, eukaryotic initiation factors (eIFs) eIF3, eIF4E, eIF4A, and eIF4G are involved in 5' cap binding, recruiting the ribosomal subunit, bind to the Poly A Binding Protein at the 3' end to circularize the mRNA [209]. Regulation of global translation occurs in part by the altering phosphorylation state of the eIFs [209]. One way to alter the eIFs' phosphorylation state is by activating the mTOR pathway. The protein complex known eIF4 Binding Proteins (or eIF4BPs) compete for binding sites in eIF4E and eIF4G and inhibit translation initiation. Activating the mTOR pathway phosphorylates the eIF4BPs, thus preventing their binding, and enable translation initiation to occur [208, 210]. Besides global regulation of translation, complex regulatory networks exist to control the translation of specific mRNAs. Regulation of translation is not solely based on the naked mRNA for a substrate, but rather the interaction of RNA-RNA and protein-RNA complexes. This mechanism of translation depends largely on specific sequences found

within the mRNA; however, the untranslated regions (UTRs) at the 5’ or 3’ end primarily contains cis regulatory elements involved in post-transcriptional gene expression [211]. These sequences can facilitate the binding of RNA binding proteins (RBPs) or adopt secondary structures that promote or prevent the translation of a given mRNA [212]. While the 5’UTR contains sequences that play a role in post-transcriptional regulation, it is clear that 3’ UTR contains a variety of regulatory elements that enables several modes of translational control at the 5’ and 3’ end [213].

Importance of the 3’UTR

The 3’UTR has shown to play a role in translational regulation through the interaction with RNA binding proteins and microRNAs [214]. Moreover, several studies have researched the biological importance of the 3’UTR by investigating its length. Computational analysis revealed that mean 3’UTR length in human transcripts is about four times the length of its 5’UTR [215, 216]. Additionally, this study revealed that the 3’UTR is longer than the 5’UTR from invertebrates to vertebrates [216]. Therefore, a given mRNA can contain multiple of regulatory sequences in the 3’UTR due to its length. Alternative polyadenylation (APA) sites found within the 3’UTR provides an example of how size of the 3’UTR matters in translational regulation. mRNA transcripts can have the same protein coding sequence, but differ in the 3’UTR sequences [214]. APA generally leads to the shortening of the 3’UTR and the consequence of that action is that other regulatory elements (such as RNA binding proteins or microRNAs) which depend on those sequences would be lost [217].

Regulation of APP by 3'UTR RNA Binding Proteins

RNA binding proteins (RBPs) interact with mRNAs to regulate stability, function, and subcellular localization [203]. The mode of interaction between the RBPs and their target mRNA is through one or more RNA binding domains, that specifically bind to sequences found in the mRNA or by protein complexes that bind to the mRNA. APP expression is regulated by several RBPs via its 3'UTR. Approximately 200 nucleotides downstream of the stop codon is a 29 nucleotide sequence that is suggested to de-stabilize APP mRNA and is the target sequence for Nucleolin [100, 101]. Nucleolin is a major nucleolar protein and it is involved in chromatin decondensation, pre-rRNA transcription and ribosome assembly, and may have roles mRNA processing [218-220] and contains RNA and DNA binding domains [221]. Nucleolin physically binds to the 3'UTR of APP and causes a decreases in APP mRNA levels, APP protein levels, and reduces Aβ levels [222].

The RNA heterogeneous nuclear ribonucleoprotein C (hnRNPC) binds to the same 29 nucleotide base element [100] in the APP 3'UTR; however, it enhances APP mRNA stability and increases APP levels [100]. hnRNP C localizes predominantly to the nucleus, but it can shuttle into the cytosol [223] suggesting that this protein can regulate mRNA stability. This protein binds with specificity to UUUUU sequences [224], thus placing its binding to the 29 nucleotide sequence in the APP 3'UTR. The fact that both nucleolin and hnRNP C both bind to the same region suggest competition for this region to decrease APP mRNA and protein levels (as with nucleolin), or enhance its mRNA a protein levels (as with hnRNP C).

Further examination of into the regulation of APP by both nucleolin and hnRNP C revealed that activation of the ERK pathway leads to an increase in the expression of both

proteins and that nucleolin first binds APP mRNA and destabilizes the mRNA. This is then followed by binding of hnRNP C where it enhances APP mRNA stability [225].

A recent report demonstrated that HuD is involved regulating APP mRNA and stability and translation [226]. HuD Is predominantly found in neuronal cells where it is involved is learning and memory [227, 228]. HuD binds to the 3'UTR of APP where it enhances its stability and translation [226]. Furthermore, transgenic mice over-expressing HuD had increased APP and Aβ levels [226]. Additionally, upon examining brain tissues from patients with AD, it revealed significant increase in HuD, APP, and Aβ levels, demonstrating a role for HuD in AD [226].

While protein-RNA interactions are involved in regulating APP translation, RBPs can interact can interact with other RBPs to regulate the translation of mRNAs by forming ribonucleoprotein particles [213]. Within the first 52 nucleotides of the APP 3'UTR is a region that stabilizes APP mRNA and is the binding site for six proteins, Rck/p54, plasminogen activator inhibitor-RNA binding protein 1 (PAI/RBP1), Y-box binding protein 1 (YB1), autoantigen La/Sjogren syndrome antigen B (La/SS-B) and elongation factor 1α (EF1a) (for review see [90]). It isn't fully understood if these proteins operate together to regulate APP or what roles these proteins have individually in regulating APP with the exception of Rck/p54. Rck/p54 overexpression was shown to increase APP levels [102].

Indeed, APP mRNA stability and translation is regulated by RBPs. The RBPs recognize specific sequences with the APP 3'UTR or form protein complexes to regulate its translation. More research is needed to further characterize the interactions of the RBPs with APP mRNA. This can be achieved be examining the RNA binding domains within

these RBPs. Mutations within the APP 3'UTR or within the RNA binding domains of those RBPs could potentially alter APP protein levels and furthermore, downstream proteolytic cleavage to generate Aβ.

Regulation of APP by microRNAs

microRNAs (miRNAs) are important regulators of mRNA translation. miRNAs are small, endogenous RNAs that negatively regulate mRNA by binding to complementary sequences primarily in the 3'UTR of its target mRNAs [103, 104]. miRNAs are transcribed RNA polymerase II and is further processed into pri-miRNA by Drosha, and is exported to cytoplasm where it becomes mature miRNA by Dicer [229]. The mature miRNA is loaded on the RNA Induced Silencing Complex (RISC), which are comprised of the Argonaut (AGO) family of proteins that guide the miRNA onto its target mRNA sequence [230]. There are two methods of negative regulation of gene expression [100] by miRNAs depending on how they bind to their targets. One method is by binding with perfect complementarity to the sequence of the mRNA [105]. When this happens, the mRNA is degraded. Binding of miRNAs with near-perfect complementarity to the mRNA which enables the mRNA to be translationally suppressed [105]. Several miRNAs have been identified to bind to the APP 3'UTR and negatively regulate APP levels [106-108]. Utilizing reporter constructs containing the intact and mutant miRNA target sequence within the APP 3'UTR, several miRNAs were reported to bind to the APP 3'UTR and negatively regulate gene expression of APP reporter constructs as well as endogenous APP levels such as mir 106a, mir 106b, mir-17, mir 20a,[106, 231], mir-101 [108], and mir-153 [232] among others.

Furthermore, it has been demonstrated that miRNAs are downregulated in AD [109-111]. Moreover, single nucleotide polymorphisms (SNPs) have been identified in miRNA binding sites which alter miRNA regulation [112, 233]. This suggests that dysregulation of APP translation via miRNAs can contributed to higher APP levels and can invariably lead to AD.

Regulation of APP by Guanine Quadruplex

Guanine rich RNA can adopt a secondary structure known as a guanine quadruplex (G-quadruplex). Formation of the G-quadruplex occurs through Hoosteen hydrogen bonding between the guanine bases and are stabilized by monovalent cations such as potassium or sodium [151]. It is predicted to be as many as 376,000 G-quadruplex forming sequences in the human genome [156]. RNA G-quadruplexes are located in 5'UTRs, coding sequences, and 3'UTRs where they regulate mRNA localization [162] and predominantly suppress mRNA translation [115, 174-176, 234]. Recently, a G-quadruplex was identified in the 3'UTR of APP and the formation of this secondary structure was potassium dependent [113]. The intact G-quadruplex sequence negatively regulates APP protein steady state levels as well as APP translation [113]. Moreover, mutations in the APP 3'UTR G-quadruplex sequence lead to higher expression of APP levels and an increase in Aβ levels [113]. Since over-expression of APP protein levels can lead to increased Aβ production, further understanding into the mechanism of how the G-quadruplex regulates APP is warranted.

Discussion

Translational control of APP can occur by a variety of regulatory elements in the 3'UTR including sequences that facilitate the binding for RNA binding proteins, miRNAs, and more recently, adopt a secondary structure. RNA binding proteins can either enhance or suppress translation of APP; whereas miRNAs are involved in translational suppression. The G-quadruplex sequence acts as a negative regulator of APP translation. With so many regulatory elements in the same region, further research is needed to understand whether the above regulatory elements compete with one another for binding sites or if they cooperatively regulate APP levels. The RNA binding protein nucleolin was reported to bind G-quadruplex sequences as well as interact with the miRNA pathway, leading to mRNA decay [235]. It is currently unknown whether nucleolin binds to the G-quadruplex or interacts with the miRNA pathway in the APP 3'UTR.

While this review focused on regulatory elements controlling the translation of APP in the 3'UTR, one cannot exclude regulatory elements in other regions of the APP mRNA. APP contains an iron response element that is involved in cap-dependent translation of APP [89] as well as an internal ribosome entry site (IRES) which promotes cap-independent translation of APP [86] in the 5'UTR. Within the coding sequence of the APP mRNA is a predicted binding site for the RNA binding protein FMRP [96] which suppresses the translation of APP. hnRNP C was shown to compete with FMRP for the same site and thus increase APP translation when bound [98]; however, it is unknown whether hnRNP C competition with FMRP is dependent on the 29 nucleotide base element in to which it binds in the APP 3'UTR.

Regulation of APP by the 3'UTR G-quadruplex warrants further investigation. The role of G-quadruplexes in translational regulation of mRNAs is poorly understood. G-quadruplexes are known to bind a variety of RNA binding proteins [193] to facilitate translational suppression of mRNAs that harbor such structures in their 3'UTR. Additionally, G-quadruplexes have been shown to contain binding sites for miRNAs [236] which demonstrates a possible synergistic interaction for controlling mRNA translation.

In summary, dysregulation of APP protein synthesis mediated by the regulatory elements found within the APP 3'UTR could generate abnormal APP protein levels, which increases the chance of producing high amounts of Aβ. While translational control of APP is quite complex, elucidating the mechanism by which cis/trans regulatory elements control it translation will shed insight into therapeutic strategies for AD.

Chapter 4

FMRP and Its Paralog FXR2P Bind to G-quadruplex in the 3'UTR of the Amyloid Precursor Protein and Regulate its Expression

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Abstract

RNA sequences rich in guanine nucleic acids can adopt non-canonical secondary structures called a guanine quadruplex, which is achieved through Hoogsteen hydrogen bonding. RNA guanine quadruplex structures have been reported to regulate different aspects of mRNA metabolism such as mRNA splicing, localization, and translation. While several reports implicate guanine quadruplexes as important regulators of translation, the mechanism by which this secondary structure influence translation remains elusive. Previous work from our lab identified a guanine quadruplex structure in the 3' untranslated region of the amyloid precursor protein. The amyloid precursor protein plays a critical role in Alzheimer's disease pathogenesis in that it can be cleaved by proteases that releases its amyloid beta region. The accumulation of amyloid beta can lead to the formation of amyloid beta plaques, a central hallmark of Alzheimer's disease. Additionally, over-expression of the amyloid precursor protein can lead to increased

production of amyloid beta. Therefore, further research into the mechanism by which the amyloid precursor protein is regulated. Our previous findings demonstrate that the amyloid precursor protein translation is negatively regulated by a guanine quadruplex in the 3' untranslated region; however, how this regulation occurs remains unknown. We present here evidence demonstrating that the Fragile X Mental Retardation Protein and its paralog Fragile X Related Protein 2 binds to the guanine quadruplex sequence in the 3' untranslated region of the amyloid precursor protein and regulate its expression.

Introduction

Deposition of amyloid-beta (Aβ) plaques and neurofibrillary tangles are molecular hallmarks of Alzheimer's disease (AD) which is the most common form of dementia. Aβ is generated from proteolytic cleavage of its precursor molecule, the amyloid precursor protein (APP), by β- and γ- secretases in the "amyloidogenic pathway". APP likely functions in synapse formation [237]; while increase in its Aβ fragment correlates with synaptic loss and dysfunction [64, 238, 239]. Additionally, increase in APP levels are implicated in the AD pathogenesis, as over-expression of APP leads to an increase in proteolytic cleavage by the amyloidogenic pathway [240, 241]. This claim is supported by research demonstrating that APP is located on chromosome 21, and individuals with Down Syndrome (trisomy 21) have increased APP levels [199, 242, 243]. Additionally, higher levels of APP are found in individuals that have the Swedish mutation [201]. In both instances, there is an increases in APP levels, as well as Aβ; moreover, these individuals develop clinical AD pathology much earlier (early-onset AD). As APP is a substrate for β-secretase, over- production of APP provides more substrate to be cleaved in the amyloidogenic pathway.

The data suggest that dysregulation of APP levels plays an important role in AD pathology. Our lab previously reported that APP contains a guanine quadruplex (G-quadruplex) which negatively regulates its translation[113]. A G-quadruplex is a secondary structure formed in RNA (as well as DNA) rich in guanine nucleic acids. The guanine bases are able to interact with one another through Hoogsteen hydrogen binding, forming guanine tetrads, and are stabilized by monovalent cations such as potassium or sodium [115, 163]. G-quadruplex structures have been shown to have important biological roles such as mRNA spicing [244], localization [162], and mRNA translation [160, 168, 174, 178]. The primary role for G-quadruplex structures involves translational suppression; however, G-quadruplexes can promote translation via cap-independent mechanisms when located near internal ribosomal entry sites (IRES) [245].
G-quadruplex structures serve as binding sites for RNA binding proteins (RBPs) which facilitate translational regulation. Several RBPs have been identified to bind to G-quadruplex structures and repress translation such as nucleolin [195, 246] and FMRP [247-249]. While we previously reported that APP translation is negatively regulated by a 3'UTR G-quadruplex in a post-transcriptional manner and that mutations in this sequence leads to increased APP and Aβ levels; however, the mechanism behind this regulation is unknown.

APP is post-transcriptionally regulated by several trans binding factors such as RNA binding proteins [250]and miRNAs [106, 231]. It is currently unknown whether or not these proteins bind to the APP 3'UTR G-quadruplex. Utilizing mass spectrometry, we

were able to identify several proteins that bind to the G-quadruplex sequence located in its 3'UTR.

We decided to investigate two proteins from our list, the Fragile X Mental Retardation Protein (FMRP) and Fragile X Related Protein 2 (FXR2P). FMRP is a ubiquitously expressed RBP that controls the translation of its target mRNAs generally by repression. A substantial trinucleotide CGG repeated in the 5'UTR of the fragile x mental retardation-1 gene leads to the repression of FMRP and causes Fragile X Syndrome, a common form of inherited mental retardation [251, 252]. FMRP contains three RNA binding domains, two KH domains and a domain rich in arginine and glycine called the RGG box [253]. The KH domains are believed to bind RNAs containing "kissing complex" motifs [254], while the RGG box has been shown to bind G-quadruplex sequences [248, 255]. APP mRNA in a target for FMRP and previous studies demonstrates APP translation is repressed by FMRP [96, 98]. However, it remains unclear whether FMRP binds to the G-quadruplex in the APP 3'UTR in order to regulation its translation.

FXR2P is a conserved paralogue of FMR has the same RNA binding domains and binds to RNAs and polyribosomes suggesting a similar function in translational repression [256-259]. Little is known about the specific mRNA targets of FXR2P; however, it has been reported that FXR2P operates with FMRP to regulate circadian rhythm by regulating clock mRNAs [260] as well as regulate glucose metabolism in mice [261]. While FXR2P contains the RGG binding domain, it has only been shown for FMRP having the ability to use this region to bind G-quadruplex sequences [256]. Furthermore, APP mRNA has not been identified as a target of FXR2P. We present here evidence that

both FMRP and FXR2P bind to the APP 3'UTR G-quadruplex and regulates APP expression.

Materials and Methods

Identification of putative APP 3'UTR G-quadruplex binding proteins

Using the CLIPdb database, we searched the APP gene on chromosome 21 (position 27252261 – 27544046 gene ID: ENSG00000142192) to determine whether proteins are predicted to bind to the APP 3'UTR G-quadruplex sequence. This sequence is located at positions 27253613 – 27253631 of chromosome 21.

Database for Annotation, Visualization, and Integrated Discovery (DAVID): In order to categorize the proteins according to their biologically relevant functional roles, we used the DAVID database to group APP 3'UTR G-quadruplex binding proteins. Details about the specifics of this program can be found in [262].

Cell Culture

Experiments were carried out in HeLa and HEK293 cells (as indicated in the Results), purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum, L-glutamine (2%), penicillin (25,000 U/ml) and streptomycin (25,000 μg/ml).

G-quadruplex pulldown experiments

RNA Oligonucleotide synthesis: RNA oligonucleotides corresponding to the wild type or mutant APP 3'UTR G-quadruplex sequence were synthesized by Integrated DNA Technologies (IDT) using the following sequences:

Wild Type: 5' GGGGCGGGUGGGGAGGGG-Biotin 3'

Mutant: 5' GGGGCGGGUGGGGAAAAA-Biotin 3'

Folding of APP 3'UTR G-quadruplex oligonucleotides

Folding of the APP 3'UTR wild type and mutant G-quadruplex oligonucleotides was performed as previously described [193].

Preparation of whole cell extracts

Confluent HeLa cell cultures were washed with phosphate buffered saline (VWR) and lysed with polysome lysis buffer ((100 mM KCl, 5 mM MgCl2, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40, 1 mM DTT, 100 U ml–1 RNasin RNase inhibitor (Promega), 2 mM vanadyl ribonucleoside complexes solution (Sigma-Aldrich) 25 μl ml–1 protease inhibitor cocktail for (Sigma-Aldrich)) on ice for 15 minutes then collected using a cell scraper.

Binding of RNA oligonucleotides to streptavidin magnetic beads

This procedure was performed using the method described in [193]. Streptavidin magnetic beads (LifeTechnologies) were initially incubated in blocking buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, Triton X-100, 0.1% (w/v) bovine serum albumin and 0.02% (w/v) tRNA from *Saccharomyces cerevisiae* (Thermo Fisher Scientific) for 1 h at 4°C with rotation. Folded 3′-biotinylated RNA oligonucleotides incubated with the streptavidin magnetic beads at 4°C overnight with rotation. The following day, 500 μl of the protein extract was then incubated with streptavidin magnetic beads overnight at 4°C

with rotation. Bound proteins were eluted from the RNA by washing the beads with increasing KCl concentrations (150mM-2M). Each fraction was concentrated by trichloroacetic acid precipitation and analyzed on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining (ThermoFisher) following the manufacturer's recommendations, or western blot analysis using (for protein confirmation experiments) using primary antibodies for FXR2P (mouse 1:5000 (Santa Cruz sc-56681) or FMRP (rabbit, 1:2000 (Sigma)) and secondary goat anti-mouse 2° Antibody (700 nm; LiCor) at 1:10,000 or goat anti-rabbit 2° Antibody (800 nm; LiCor) at 1:10,000.

Mass Spectrometry Analysis

This procedure was performed by the Wistar Institute's Proteomics Facility (University of Pennsylvania) Samples from the 1M and 1.5M elutions from the pull down experiments were subjected to 10% SDS-PAGE that was allowed to run 0.5 cm gel. The lanes cut out and digested with trypsin and analyzed the digests by LC-MS/MS on a Q Exactive Plus mass spectrometer using a 95min gradient. MS/MS spectra generated from the LC-MS/MS runs were searched using full tryptic specificity against the UniProt human database (www.uniprot.org) using the MaxQuant 1.5.2.8 program. Protein quantification was performed using unique+razor peptides. False discovery rates for protein and peptide identifications were set at 1%.

Transfections

One day before transfection HEK293 cells were seeded in 6 well plates so that they would be 80% confluent. The next day, transfection of the plasmids was carried out using XtremeGene HP (Sigma-Aldrich) following the manufacturer's recommendation for

Turbofect (ThermoScientific) according the manufacturer's recommendations for single transfection (**Figure 4.4**) or co-transfections (**Figure 4.5**).

Western Blot Analysis

24 hours post transfection, cells were lysed in RIPA cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing 1X protease inhibitor cocktail (Thermo Scientific). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. The resulting supernatants were transferred to a new microcentrifuge tube and the cell lysate protein concentration was determined using the BCA protein assay kit (Pierce) according to the manufacturer's instructions.

Equal quantities of protein (~40 μg) were mixed with loading buffer and loaded into the wells of 4–12% Bis-Tris polyacrylamide gels (Invitrogen) along with molecular weight standard (LiCor). Gels were run using MES running buffer and transferred to PVDF membrane (Immobilon PSQ, Millipore) using a semi-dry transfer apparatus (Owl Scientific) and NuPage transfer buffer (Invitrogen). After transfer, membranes were blocked with Odyssey Blocking Buffer (LiCor) for 30 minutes. Next, the blocking buffer was removed and the membrane probed overnight at 4°C with blocking buffer containing A8717 (Sigma) which recognize APP (**Figure 4.4**) (rabbit, 1:1000) or Myc antibody 9B11 (**Figure 4.5**) (Cell Signaling) (mouse, 1:5000) and an antibody to β-actin (Sigma; 1:10,000). For Figure 5, a GFP antibody (Invitrogen) used to detect FMRP tagged with GFP (rabbit, 1:2000) and FXR2P (mouse 1:5000 (Santa Cruz sc-56681) antibody Membranes were washed with 1X PBST for 5 minutes (4 times). After the washes, the membrane was probed for 1 hour at room temperature with goat anti-mouse 2° Antibody

(700 nm; LiCor) at 1:10,000 diluted in blocking buffer or goat anti-rabbit 2° Antibody (800 nm; LiCor) at 1:10,000.

Statistical Analysis

All experiments were repeated 3 times unless stated otherwise. All errors are shown as standard error of the mean. Equal variance was assumed for the two-sample student's t-test (95% confidence interval). Q test was used to reject outliers at the 95% confidence interval. * indicates a $P < 0.05$, ** indicates a $P < 0.01$, and *** indicates a $P < 0.001$.

Results

Identification of APP 3'UTR G-quadruplex Binding proteins

G-quadruplex structures interact with RNA binding proteins that assist in translational regulation. In order to investigate potential proteins that bind to the APP 3'UTR G-quadruplex, we performed a bioinformatic search of the APP 3'UTR for putative binding proteins. Using the CLIPdb data base, which attempts to ascertain the nucleotide binding sequences recognized by RNA binding protein-RNA interactions from publicly available CLIP-Seq data sets [263], in order to identify proteins that bind to the APP mRNA located on chromosome 21 (ENSG00000142192). Only 4 proteins were shown to recognize sequences in the APP 3'UTR that overlap with the G-quadruplex sequence (Table 1). Of the proteins that were predicted to bind to the APP 3'UTR containing the G-quadruplex, AGO 2 was an interesting find because it is a major component of microRNA Induced Silencing Complex (miRISC) pathway [230] and microRNAs have been shown to regulate APP levels [106, 231]. However, we wanted to confirm the results from the bioinformatic search using an alternative approach as G-quadruplex

structures may not crosslink very well with current HITS-CLIP or Par-CLIP methods [248].

We next decided to perform a similar experiment used by von Hacht and colleagues to identify G-quadruplex binding proteins [193] by using the G-quadruplex sequence as bait to capture proteins. We synthesized RNA oligonucleotides (oligos) containing the intact APP 3'UTR G-quadruplex or the mutant G-quadruplex (previously described). Both oligos contained a 3'Biotin molecule which we used in our pulldown experiments (**Figure 4.1A**). We were able to bind our APP 3'UTR G-quadruplex oligos to streptavidin magnetic beads and used HeLa lysates to identify proteins that bound to wither the wild type or mutant G-quadruplex oligo. Following incubation of the HeLa lysates with either G-quadruplex oligo, proteins were eluted with increasing potassium concentration and the eluates were subjected to SDS-PAGE followed by silver staining. Figure 4.1B is a silver stain of proteins that bound to either the wild type (WT) or mutant (Mut) G-quadruplex sequence. We were specifically looking for differences in the bands from the silver stain in the WT or Mut elution lanes, or bands that were of a higher intensity in the WT elution lanes compared to the Mut elution lanes. The results indicate that are differences between elution lanes for both WT and Mut G-quadruplex lanes (indicated by the arrows in **Figure 4.1B**), suggesting that there are indeed differences in protein binding between the WT and Mut APP 3'UTR 3'G-quadruplex sequence.

In order to identify which proteins were present in the WT and Mut elutions, we sent samples from WT and Mut 1 Molar (n=2) elutions for mass spectrometry analysis. Table 2 is a list of the proteins identified from mass spectrometry. We focused on the proteins

the favored binding to the WT APP 3'UTR sequence, as the Mut sequence may not accurately represent proteins binding to that sequence due to the fact that we created the mutated sequence the Mut and it may not reflect a relevant biological interaction. We selected proteins that were identified in both mass spectrometry analysis and arranged them in order of their intensity fold change (Table 4.2 columns 1,2,3). To determine which proteins bound to the wild type APP 3'UTR G-quadruplex sequence, we examined the intensity fold change of each protein from each experiment. If the intensity fold change was greater than 1 for each experiment, we determined that protein to bind to the wild type sequence (Table 4.2 column 4). More than 1500 proteins identified by mass spectrometry, ~5% of the proteins identified favored binding to the wild type (WT) APP 3'UTR G-quadruplex sequence. Surprisingly, the only protein that was confirmed by mass spectrometry from the initial CLIPdp data base was NUDT21 (Table 1). The protein which had the highest values from two rounds of mass spectrometry analysis was the Fragile X Related Protein 2 (FXR2P). Interestingly, its paralog, the Fragile X Mental Retardation Protein (FMRP) was identified on our list and these two proteins were selected for further analysis.

Confirmation of FMRP and FXR2P binding to APP 3'UTR G-quadruplex

Our mass spectrometry analysis revealed the binding of FMRP and its paralog FXR2P to the APP 3'UTR G-quadruplex sequence. FMRP has been shown to bind G-quadruplex structures; however, FXR2P has not been shown, at least to our knowledge, to bind to G-quadruplex sequences. In order to confirm the findings from our mass spectrometry analysis, we performed the same pulldown procedure using our biotinylated APP 3'UTR G-quadruplex oligos; however, after SDS-PAGE, we performed western blot analysis and

detected FMRP and FXR2P using antibodies that recognize those proteins. Figure 4.3 indicates that both FMRP (**Figure 4.2A**) and FXR2P (**Figure 4.2B**) favors binding to the intact APP 3'UTR G-quadruplex sequence. This can be due to the fact that the intact APP 3'UTR G-quadruplex sequence is folds into this secondary structure whereas the mutant sequences does not adopt this structure[113]. This data confirms our mass spectrometry analysis and suggests that the binding of FMRP and FXR2P to the APP 3'UTR G-quadruplex may have a role in regulating APP expression.

FXR2P Regulates APP Expression

FMRP negatively regulates endogenous APP translation and has been previously reported [96, 98]; however, it is currently unknown whether FXR2P is involved in regulating APP levels. Given our previous results demonstrating FXR2P binds to APP 3'UTR G-quadruplex, we wanted to investigate whether FXR2P can regulate APP levels. We over-expressed FXR2P in HEK 293 cells to determine if there was any effect on endogenous APP levels. FXR2P over-expression was able to reduce APP levels when compared to mock transfected cells indicated by western blot analysis (**Figure 4.3A**). Upon quantification, over-expression of FXR2P lead to a .33-fold decrease in in endogenous APP levels which was statistically significant ($p=0.001108$) (**Figure 4.3B**). This is the first report demonstrating FXR2P regulates APP levels. This data suggests thatFXR2P has a role is regulating APP expression and warrants further investigation.

Preliminary Data: FMRP and FXR2P regulates APP Expression via G-quadruplex

Regulation of APP by FMRP was demonstrated to occur by FMRP binding to a guanine rich sequence in the coding sequence of the APP mRNA [96]. Putative binding sites in the APP mRNA coding sequence were identified by HITC-CLIP experiments [94] and PAR-CLIP experiments [95]. While studies identify FMRP binding to the APP coding sequence, our data suggests that FMRP can bind to the APP 3'UTR G-quadruplex sequence. Additionally, the involvement FXR2P in regulating APP levels has not been investigated until now. Given the ability of both FMRP and FXR2P to bind to the G-quadruplex sequence and that these proteins can regulate APP levels, we wanted to investigate whether regulation of APP expression by FMRP and FXR2P is mediated through the APP 3'UTR G-quadruplex. In order to address our question, we used a reporter construct containing the APP coding sequence fused to a c-Myc epitope tag as well as the APP 3'UTR sequence containing the intact or mutated G-quadruplex (previously described), we co-transfected the APP reporter constructs with a GFP tagged FMRP plasmid or FXR2P plasmid in HEK 293 cells. Our preliminary results indicate that co-expression of our APP reporter construct harboring the intact G-quadruplex sequence with either FMRP or FXR2P reduces APP expression; however, that reduction is lost upon co-expression of FMRP or FXR2P with the APP reporter construct containing the mutant G-quadruplex sequence (**Figure 4.4**). Although this is preliminary data warrants additional experiments to determine the overall regulatory effect, it demonstrates for the first time that both FMRP and FXR2P may regulate APP expression through its G-quadruplex sequence in the 3'UTR.

Discussion

We previously identified the presence of a G-quadruplex sequence in the 3'UTR of APP and further demonstrated that it negatively regulates APP translation in a post-transcriptional manner. In this report, we sought out to identify proteins that bind to this G-quadruplex sequence in order to further characterize how the G-quadruplex mediates translational regulation of APP. We report here our findings in which we identified 78 putative RNA binding proteins from our mass spectrometry analysis that favor the binding to the intact APP 3'UTR G-quadruplex sequence. Of those 78 putative proteins, FMRP and FXR2P were selected for further investigation. Our results indicate, for the first time, that both FMRP and FXR2P bind to the APP 3'UTR G-quadruplex sequence. Furthermore, we report for the first time that FXR2P over-expression reduces APP expression. Lastly, we report preliminary data, which for the first time, indicates that FMRP and FXR2P reduce APP expression mediated by the 3'UTR G-quadruplex; however, additional experiments are needed to confirm this finding.

FMRP and FXR2P contain conserved RNA binding domains such as the two KH domains and the RGG box. While FXR2P has not been shown to bind G-quadruplex sequence, FMRP has been shown to bind to such sequences through its RGG box domain [248, 264, 265]. Addressing whether FMRP, or FXR2P, uses its RGG box domain to bind to the APP 3'UTR G-quadruplex in order to regulate APP expression warrants further investigation. Additionally, it was reported that FMRP binds to the coding sequence of APP mRNA [96] and we cannot definitively rule out whether the guanine rich region in the coding sequence of APP alone is necessary for FMRP to regulation APP expression. Our preliminary data suggests that FMRP regulates APP expression

through the 3'UTR G-quadruplex, which may suggest that FMRP may selectively or preferentially use the G-quadruplex sequence to regulate APP expression, however, further experiments are needed to draw this conclusion.

FMRP is a known regulator of translation and several modes of translational control by FMRP has been proposed. One model suggests that FMRP regulates mRNA translation by targeting translation initiation. This is achieved by FMRP recruiting CYFIP1 protein which then interacts with eIF4G protein, preventing its binding to the 5' cap, thus inhibiting cap-dependent translation [266]. The next model for translational control by FMRP involves the miRNA pathway. FMRP was shown to interact with proteins involved in the miRNA pathway such as Argonaut 2 [267]. This would suggest that FMRP could regulate a subset of mRNAs through the miRNA pathway, which has been shown for PSD-95 [268]. Lastly, FMRP was shown to stall translation elongation by forming complexes that contained ribosomal proteins, mRNA, and additional proteins [94]. Later studies showed that FMRP binds directly to the ribosome, demonstrating that the KH domain could bind in between the large and small ribosomal subunits, thus stalling translation [269]. It is currently unclear how FMRP regulates APP translation and further experiments are needed to determine this mechanism

Both FMRP and FXR2P seem to have the ability to reduce APP levels; however, it remains to be determined if they regulate APP levels cooperatively or independent of one another. One study reported that FMRP and FXR2P in a cooperative manner in order to regulate circadian rhythm [260]. Loss of FMRP and FXR2P in mice results in abnormal circadian rhythm due to dysregulation of CLOCK mRNAs in fmr1/fxr2 double KO mice

[260]. Circadian rhythm is dysregulated in AD [270, 271]. In addition, over-expression of APP levels leads to increased wake duration and decreased sleep as observed in transgenic mouse models over expression APP [272, 273]. As over-expression of APP leads to increased Aβ levels, studies have also demonstrated that increased Aβ deposition caused a decrease in sleep efficiency [274, 275]. Given our results indicating APP expression is regulated by FMRP and FXR2P, it might be of interest to further investigate this interaction and regulation in circadian rhythm.

Our mass spectrometry results yielded several proteins that could interact with the intact APP 3'UTR G-quadruplex (Table 2). Further experiments are needed to determine if there are additional proteins that bind to this sequence and explore additional mechanisms of regulation. G-quadruplex sequences can target the initiation step in translation. One possible way this can be achieved is by proteins binding to the sequence and thus interfere with eukaryotic initiation factors in order to suppress translation or by sequestering these initiation factors to promote translation by cap-independent translation. Our results indicate that further exploration is needed to fully characterize how the APP 3'UTR G-quadruplex mediates translational control of APP expression and may provide additional insight into the regulation of APP and how it is involved in AD pathogenesis.

Figure and Table Legends

Figure 4.1: Identification of APP 3'UTR G-quadruplex binding proteins: (A) Schematic of the procedure used to identify APP 3'UTR G-quadruplex binding proteins. APP 3'UTR wild type or mutant RNA G-quadruplex oligos were synthesized, each biotinylated at the 3' end of the sequence. The G-quadruplex oligos were then used to capture proteins from HeLa lysates. The eluted proteins were then used for downstream applications such as silver staining and mass spectrometry analysis. (B) Silver stain of the SDS-PAGE gel from the eluted proteins. The proteins were eluted by increasing KCl [1M-1.5M] concentration. (WT)= wild type G-quadruplex sequence, (Mut)= mutant G-quadruplex sequence. The beads lanes were used as a control since no RNA oligo was used to bind to the streptavidin magnetic beads; therefore, no proteins were eluted in these lanes. Arrows indicate differences in band intensities between the WT and Mut lanes.

Figure 4.2: Confirmation of FMRP and FXR2P binds to the APP 3'UTR G-quadruplex sequence: FMRP and FXR2P were selected from our mass spectrometry list to confirm whether these proteins bind to the APP 3'UTR G-quadruplex sequence. Following the same procedure used in Figure 4.1A, only this time western blot analysis was performed following KCl [500mM -1.5M] elution of proteins and probed for (A) FMRP or (B) FXR2P. Asterisk indicates the expected migration of the target protein.

Figure 4.3: FXR2P over-expression reduces endogenous APP levels: FXR2P was over-expressed in HEK 293 cells. Following transfection, HeLa lysates were collected and used in SDS-PAGE followed by western blot analysis (A) Top gel is the probe for APP, middle lane is the probe for FXR2P, and the bottom gel is the probe for β-actin

which was used as a loading control. (B) Quantification of the western blot in (A). Y-axis represents the APP levels normalized to β-actin. X-axis represents the samples containing FXR2P (+FXR2P) over-expression or without FXR2P over-expression (-FXR2P) (n=4) p= 0.001108.

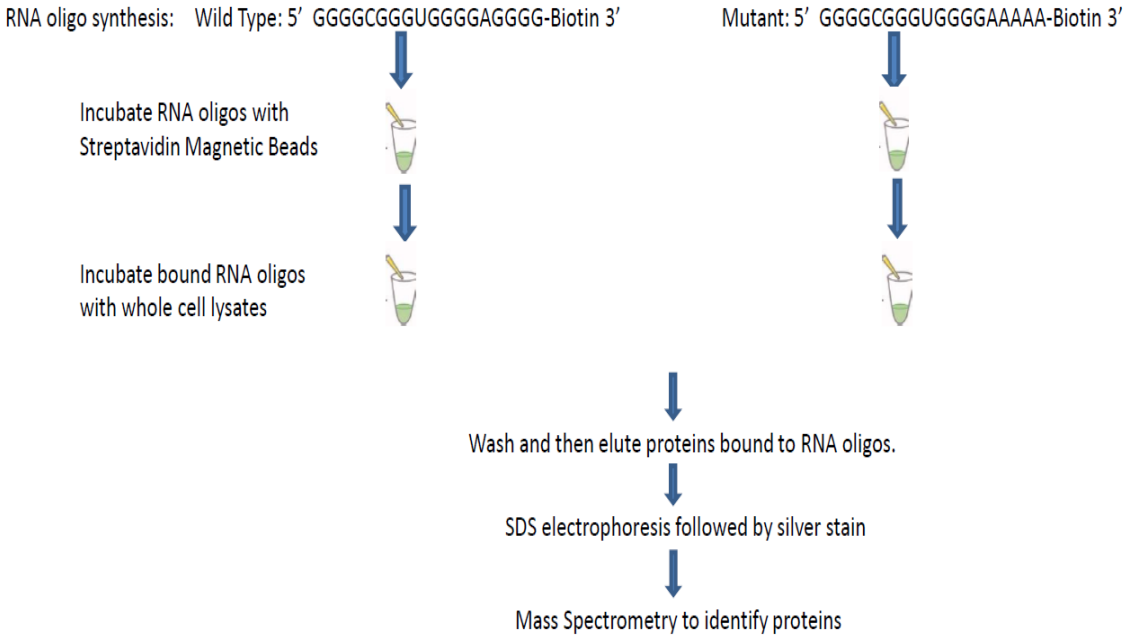
Figure 4.4 Preliminary Data: FMRP and FXR2P regulates APP expression via APP 3'UTR G-quadruplex: GFP-tagged FMRP and FXR2P were co-transfected with APP-Myc_3'UTR reporter constructs harboring either the wild type or mutant G-quadruplex in HEK 293 cells. Following transfection, lysates were collected, subjected to SDS-PAGE followed by western blot analysis. Top gel is the probe for APP using a Myc antibody, followed by the probe for β-actin (used as a loading control), followed by FXR2P probed gel, and lastly, the gel probed for FMRP. Lanes 1 and 4 control lanes that were not co-transfected with wither FMRP or FXR2P.

Table 1: Bioinformatic approach to identify putative APP 3'UTR G-quadruplex sequence: Using the CLIPdb database, we searched the APP gene on chromosome 21 to determine whether proteins are predicted to bind to the APP 3'UTR G-quadruplex sequence. This sequence is located at positions 27253613 – 27253631 of chromosome 21. The gene symbols and their target sequence location are listed.

Table 2: Putative APP 3'UTR G-quadruplex binding proteins Identified by mass spectrometry: Proteins listed in this table indicate potential binding proteins to the APP 3'UTR G-quadruplex. Listed are the gene names that were identified as well as the

intensity fold change between each experiment. Genes are listed according to the values of the intensity fold change.

Figure 4.1
(A)


RNA oligo synthesis: Wild Type: 5′ GGGGCGGGUGGGGAGGGG-Biotin 3′
Mutant: 5′ GGGGCGGGUGGGGAAAAA-Biotin 3′
Incubate RNA oligos with Streptavidin Magnetic Beads
Incubate bound RNA oligos with whole cell lysates
Wash and then elute proteins bound to RNA oligos.
SDS electrophoresis followed by silver stain
Mass Spectrometry to identify proteins

(B)

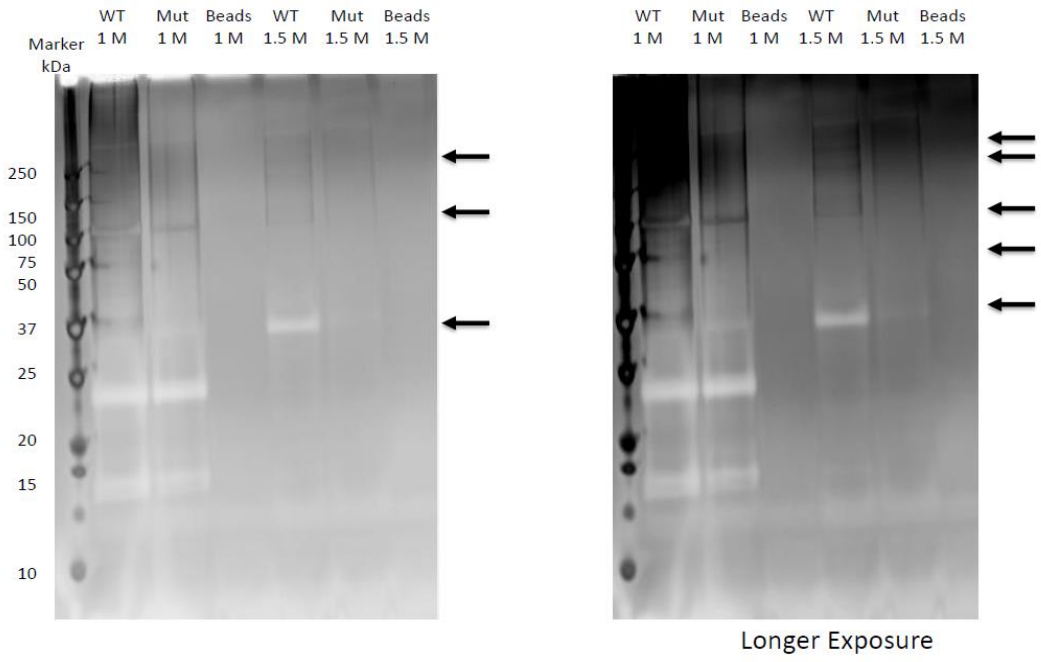
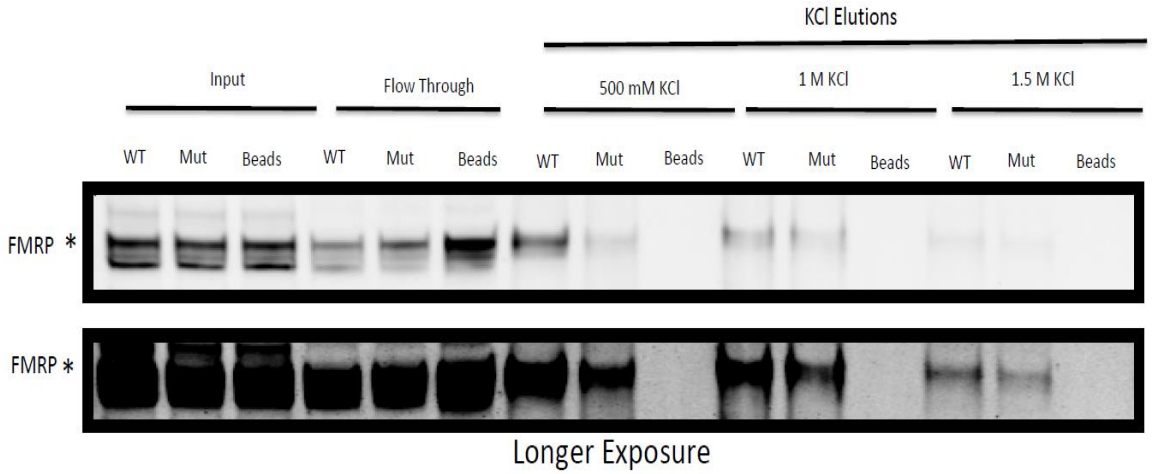

Marker kDa
WT 1 M
Mut 1 M
Beads 1 M
WT 1.5 M
Mut 1.5 M
Beads 1.5 M
250
150
100
75
50
37
25
20
15
10
Longer Exposure

Figure 4.2
(A)


KCl Elutions
Input
Flow Through
500 mM KCl
1 M KCl
1.5 M KCl
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FMRP *
FMRP *
Longer Exposure

(B)

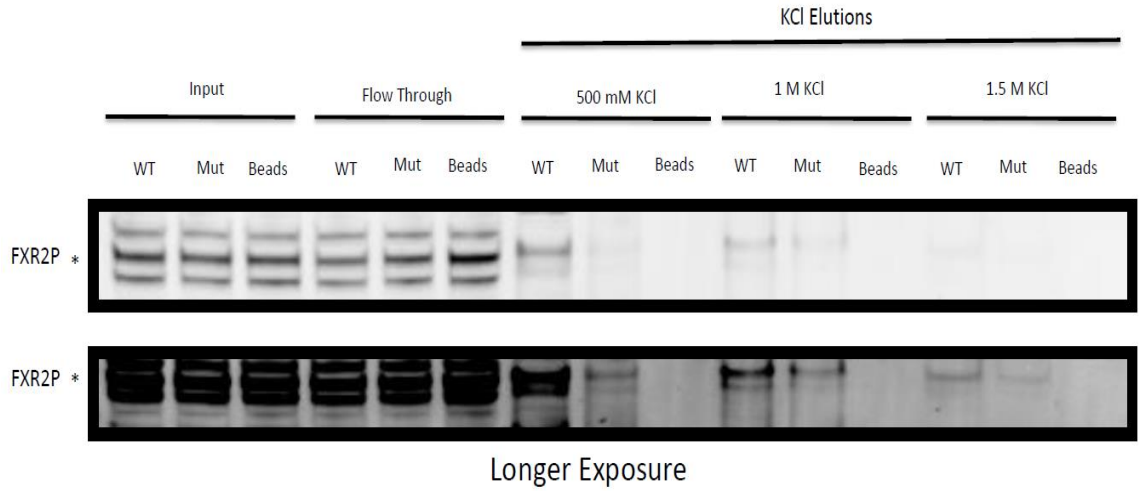
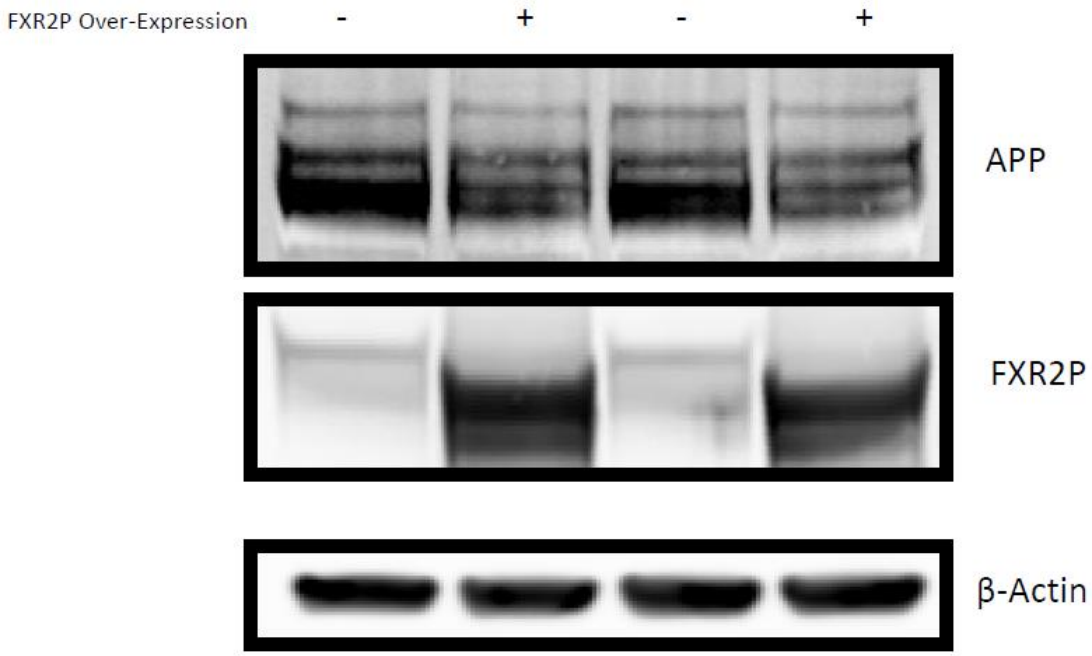

KCl Elutions
Input
Flow Through
500 mM KCl
1 M KCl
1.5 M KCl
WT Mut Beads WT Mut Beads WT Mut Beads WT Mut Beads WT Mut Beads
FXR2P *
FXR2P *
Longer Exposure

Figure 4.3
(A)


FXR2P Over-Expression
-
+
-
+
APP
FXR2P
β-Actin

(B)

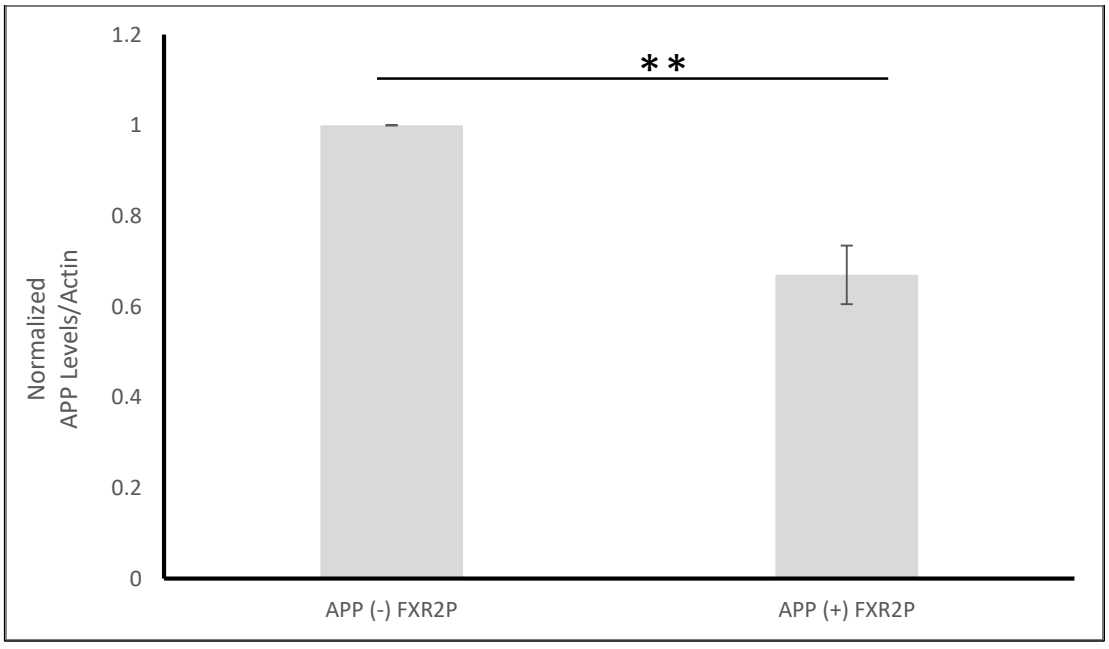
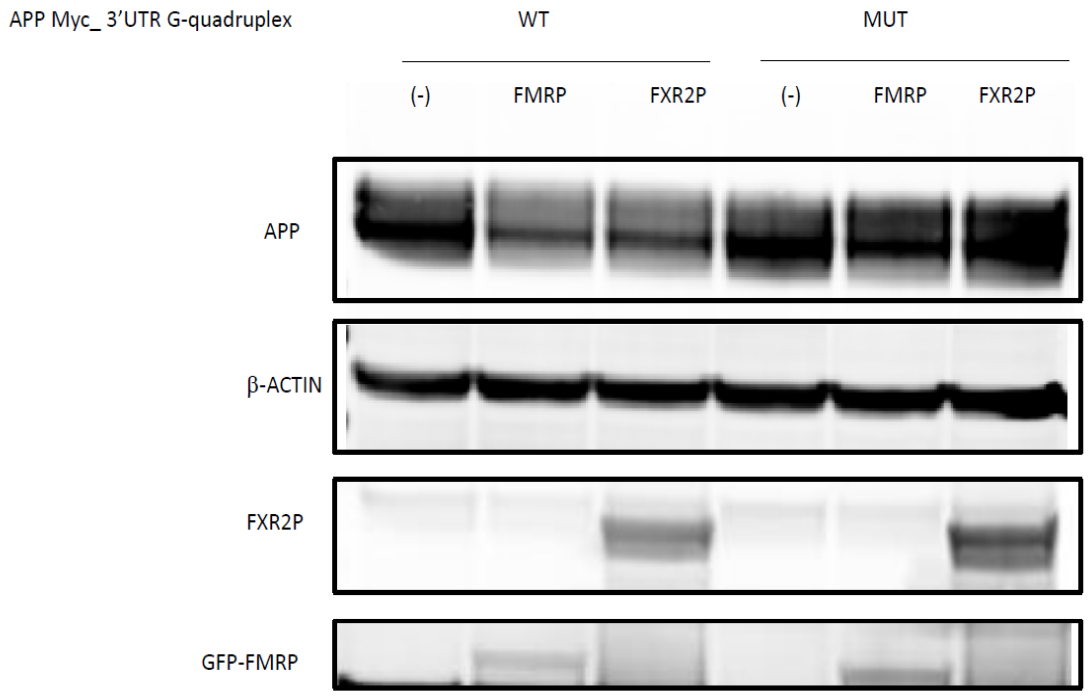

1.2
1
0.8
0.6
0.4
0.2
0
Normalized APP Levels/Actin
**
APP (-) FXR2P
APP (+) FXR2P

Figure 4.4


APP Myc_ 3'UTR G-quadruplex
WT
MUT
(-)
FMRP
FXR2P
(-)
FMRP
FXR2P
APP
β-ACTIN
FXR2P
GFP-FMRP

Tables

Table 4.1

APP 3'UTR G-Quadruplex Location on Chromosome 21: 27253613 – 27253631

Gene Symbol	Binding Region on Chromosome 21
AGO 2	21: 27253620-27253640
LIN28A	21: 27253620-27253640
Mov-10	21: 27253540 - 27253620
NUDT21	21: 27425620-27425640

Table 4.2

Proteins found in Experiments 1 and 2	Intensity Fold Change WT/Mut Experiment 1	Intensity Fold Change WT/Mut Experiment 2	WT>MUT	MUT>WT
FXR2	235990000.0	1.4	FXR2	--
NXF1	123580000.0	5.5	NXF1	--
LSM14A	61421000.0	2.3	LSM14A	--
RRP1	23769000.0	0.0	--	--
PEG10	17150000.0	1.1	PEG10	--
HNRNPR	11156000.0	1.3	HNRNPR	--
UPF3B	2213700.0	2.0	UPF3B	--
FXR1	121.6	-1.9	--	--
ALYREF	79.2	1.9	ALYREF	--
EIF4G2	54.4	1.4	EIF4G2	--
PRPF31	46.6	3.8	PRPF31	--
SCAF11	40.8	5.6	SCAF11	--
SART1	40.5	1.9	SART1	--
CCAR2	40.5	1.5	CCAR2	--
DYNC1H1	32.1	-1.0	--	--
UBAP2L	24.8	1.8	UBAP2L	--
SND1	23.7	-1.6	--	--
MRPS23	16.2	-2641800.0	--	--
MEPCE	14.5	-22626000.0	--	--
ILF3	12.2	1.2	ILF3	--
SERBP1	11.7	1.5	SERBP1	--
AKAP8	11.2	2412600.0	AKAP8	--

LARP1	11.1	1.3	LARP1	--
ZNFX1	10.8	10065000.0	ZNFX1	--
STRBP	10.4	16996000.0	STRBP	--
EIF4G1	10.3	3.6	EIF4G1	--
CCAR1	8.7	5.8	CCAR1	--
EEF1A1	8.4	2.6	EEF1A1	--
DDX18	8.3	-1.0	--	--
LARP4B	8.2	-2.0	--	--
NUDT21	8.1	23.9	NUDT21	--
SART3	8.0	11.3	SART3	--
SNRPD1	7.9	-1.1	--	--
DDX5	7.8	1.1	DDX5	--
FAM120A	7.6	1.4	FAM120A	--
TCERG1	7.5	-1.3	--	--
HNRNPA1	7.4	1.5	HNRNPA1	--
MTHFSD	7.4	2.0	MTHFSD	--
YTHDC2	7.3	1.7	YTHDC2	--
CIRBP	7.3	1.2	CIRBP	--
POLR2A	7.3	1.5	POLR2A	--
ILF2	7.3	1.0	ILF2	--
POLR2B	7.2	1.1	POLR2B	--
HNRNPA0	7.2	1.4	HNRNPA0	--
DHX9	7.1	1.4	DHX9	--
HNRNPL	7.0	1.6	HNRNPL	--
RBM14	6.9	1.3	RBM14	--
SAFB	6.8	-11445000.0	--	--
UTP14A	6.5	-814410.0	--	--

LARP4	6.5	-6.5	--	--
PABPN1	6.0	1.6	PABPN1	--
ATXN2L	6.0	-2.0	--	--
G3BP2	5.9	1.6	G3BP2	--
NOP14	5.9	0.0	--	--
ZCCHC6	5.6	1.5	ZCCHC6	--
MOV10	5.6	-1.2	--	--
ZFR	5.5	1.6	ZFR	--
RALY	5.4	-5.2	--	--
HDLBP	5.3	-2.6	--	--
CNOT1	5.1	8.2	CNOT1	--
FMR1	4.9	1.2	FMR1	--
NOP2	4.9	-1.8	--	--
NSUN2	4.9	3.0	NSUN2	--
USP10	4.8	-2.6	--	--
SUGP2	4.7	0.0	--	--
EXOSC10	4.7	-1.6	--	--
SYNCRIP	4.7	1.2	SYNCRIP	--
ADAR	4.7	-2.2	--	--
SRPK2	4.6	-1.1	--	--
ZNF326	4.6	3.4	ZNF326	--
RBM39	4.6	-4.6	--	--
DDX3X	4.5	1.1	DDX3X	--
SF3B2	4.4	1.5	SF3B2	--
HNRNPU	4.3	-1.5	--	--
PRKDC	4.2	-1.1	--	--
RAN	4.2	1.6	RAN	--

NOP9	4.2	-1.2	--	--
RRP8	4.2	1.2	RRP8	--
HNRNPC	4.1	-1.5	--	--
EEF2	4.0	-3.3	--	--
PTBP1	3.7	-1.7	--	--
SNRNP200	3.7	1.1	SNRNP200	--
IGF2BP3	3.6	-1.0	--	--
TRIM56	3.6	1.6	TRIM56	--
NUFIP2	3.6	-2.1	--	--
NOP56	3.6	-2.3	--	--
GTPBP4	3.6	-42.6	--	--
NCL	3.5	1.8	NCL	--
FUS	3.5	5.4	FUS	--
DDX17	3.5	-1.2	--	--
PRPF8	3.4	-1.3	--	--
XRN2	3.4	1.0	XRN2	--
RBMX	3.4	-2.5	--	--
WDR46	3.3	0.0	--	--
ZC3H4	3.3	10.0	ZC3H4	--
RPS20	3.3	-2.2	--	--
SF3B4	3.2	1.8	SF3B4	--
DDX6	3.2	-1.9	--	--
MATR3	3.2	1.5	MATR3	--
ZC3H15	3.2	-1.5	--	--
DNTTIP2	3.2	0.0	--	--
EIF2S2	3.1	-1.5	--	--
SF3B1	3.1	1.1	SF3B1	--

AGO2	3.0	-1.2	--	--
AGO3	3.0	-4576000.0	--	--
RPL10A	3.0	-7.7	--	--
TFAM	2.9	4.1	TFAM	--
RPS10	2.8	-24.5	--	--
SUPT5H	2.8	4.9	SUPT5H	--
PUF60	2.8	-1.3	--	--
CAPRIN1	2.7	-1.2	--	--
PRDX1	2.7	-1.2	--	--
KRR1	2.6	0.0	--	--
EIF2S1	2.6	-1.5	--	--
ZNF598	2.5	-1.3	--	--
RPS7	2.5	-4.9	--	--
FBL	2.5	-14.3	--	--
PSIP1	2.5	1.5	PSIP1	--
EIF3A	2.4	4.7	EIF3A	--
ELAVL1	2.4	3.1	ELAVL1	--
DDX51	2.4	-1.0	--	--
ZC3HAV1	2.4	-1.5	--	--
PABPC1	2.4	-3.1	--	--
PES1	2.3	-2.3	--	--
PURB	2.2	2.0	PURB	--
RPS4X	2.1	-12.0	--	--
RPS3	2.1	-5.3	--	--
REXO4	2.0	-10.0	--	--
EIF4B	2.0	-1.0	--	--
EIF3C	2.0	1.6	EIF3C	--

DHX57	2.0	-2.3	--	--
RBM19	2.0	-1.7	--	--
EIF3D	1.9	4.4	EIF3D	--
EIF3L	1.9	1.6	EIF3L	--
DDX24	1.9	-4.2	--	--
POLRMT	1.9	0.0	--	--
PURA	1.9	-1.9	--	--
DIMT1	1.9	-2.8	--	--
KIAA0020	1.9	-12.7	--	--
FTSJ3	1.8	0.0	--	--
RBM3	1.8	1.3	RBM3	--
NOP58	1.8	-2.7	--	--
DDX21	1.8	-6.7	--	--
LAS1L	1.8	-1051600.0	--	--
FAM98A	1.8	-3.4	--	--
CCDC86	1.8	-41.0	--	--
RPS3A	1.7	-4.7	--	--
PUS7	1.7	-1.9	--	--
NIFK	1.7	-19513000.0	--	--
UTP20	1.7	0.0	--	--
DDX1	1.7	-1.4	--	--
HNRNPM	1.7	1.0	HNRNPM	--
PABPC4	1.7	-2.7	--	--
RBM34	1.7	-19297000.0	--	--
PWP2	1.7	-4.0	--	--
RRP1B	1.7	-20.5	--	--
SRSF7	1.7	-1.3	--	--

ABCF1	1.6	-1.2	--	--
CEBPZ	1.6	-41446000.0	--	--
STRAP	1.6	-2.4	--	--
LARP7	1.6	-1.6	--	--
NKRF	1.6	-2.3	--	--
U2AF2	1.5	1.4	U2AF2	--
RPL30	1.5	-32.5	--	--
UPF1	1.5	-2.2	--	--
NOC2L	1.5	-6551400.0	--	--
UTP3	1.5	-8002700.0	--	--
RPL22	1.5	-3.5	--	--
EIF4A3	1.5	3.9	EIF4A3	--
HNRNPAB	1.5	-1.3	--	--
GNL3	1.4	0.0	--	--
EIF3G	1.4	1.2	EIF3G	--
SRP14	1.4	1.3	SRP14	--
PUS1	1.4	-1.8	--	--
RTCB	1.4	-1.9	--	--
DDX50	1.3	-1772400.0	--	--
PARP1	1.3	-1.5	--	--
DDX27	1.3	-91147000.0	--	--
NOC3L	1.3	-4682100.0	--	--
RSL1D1	1.3	-104500000.0	--	--
RPL14	1.3	-350380000.0	--	--
NOLC1	1.3	1.2	NOLC1	--
SRSF9	1.2	-3545800.0	--	--
SRSF1	1.2	2.3	SRSF1	--

CSDE1	1.2	-14.0	--	--
AQR	1.1	-3.3	--	--
NPM1	1.1	-2.4	--	--
NOP16	1.1	0.0	--	--
NSUN5	1.1	-2.2	--	--
RBM28	1.1	-26569000.0	--	--
TOP1	1.1	-7.2	--	--
NGDN	1.1	-12583000.0	--	--
ANKHD1	1.1	1.2	ANKHD1	--
BMS1	1.1	0.0	--	--
IMP3	1.1	0.0	--	--
PPAN	1.0	-17821000.0	--	--
MPHOSPH10	1.0	-4931900.0	--	--
RPL5	1.0	-3.5	--	--
DDX10	-1.0	-4373500.0	--	DDX10
RPL15	-1.0	-14.1	--	RPL15
HNRNPUL2	-1.0	-2944000.0	--	HNRNPUL2
DDX31	-1.1	0.0	--	--
ABT1	-1.1	-2119500.0	--	ABT1
NOL10	-1.1	-5248200.0	--	NOL10
RPL4	-1.1	-15.6	--	RPL4
DHX36	-1.1	-4.4	--	DHX36
RPS26	-1.1	-19.2	--	RPS26
NAT10	-1.2	-5.5	--	NAT10
RPL35A	-1.2	-10667000.0	--	RPL35A
YTHDF2	-1.2	-1.8	--	YTHDF2
RPS15A	-1.2	-5.2	--	RPS15A

RPLP0	-1.3	-7.4	--	RPLP0
RPS2	-1.3	-5.2	--	RPS2
U2AF1	-1.3	4.2	--	--
TCOF1	-1.4	1.4	--	--
RPL27	-1.4	-20.9	--	RPL27
RRP12	-1.4	0.0	--	--
GRWD1	-1.5	-1.4	--	GRWD1
IGF2BP1	-1.5	-1.8	--	IGF2BP1
RPL8	-1.6	-6.1	--	RPL8
RPL21	-1.6	0.0	--	--
RPL7	-1.6	-24675000.0	--	RPL7
RPL17	-1.7	-150480000.0	--	RPL17
RPS24	-1.8	-7137600.0	--	RPS24
RPL18A	-1.8	-560530.0	--	RPL18A
XRCC6	-1.8	-38.4	--	XRCC6
SURF6	-1.9	0.0	--	--
RPL23A	-1.9	-320340000.0	--	RPL23A
RPL10	-1.9	0.0	--	--
RPL24	-2.0	0.0	--	--
SSB	-2.1	-1.2	--	SSB
YBX1	-2.4	-24.2	--	YBX1
GNL2	-2.4	0.0	--	--
PAPD5	-2.5	0.0	--	--
RPL29	-2.5	-4.3	--	RPL29
EIF5B	-2.6	-1.6	--	EIF5B
DHX15	-3.3	-8.2	--	DHX15
GRSF1	-3.5	-1.4	--	GRSF1

RBM4	-3.6	-3.4	--	RBM4
BRIX1	-4.0	31.5	--	--
RPS8	-4.4	-6946000.0	--	RPS8
GTF2F1	-4.6	-1.2	--	GTF2F1
EBNA1BP2	-5.6	0.0	--	--
HNRNPH1	-6.9	-3.8	--	HNRNPH1
RPF2	-7.4	-2493000.0	--	RPF2
DDX54	-8.6	0.0	--	--
RPL3	-17.6	-86988000.0	--	RPL3
HNRNPH2	-92704000.0	-13749000.0	--	HNRNPH2

Chapter 5

Summary of Research, Future Directions, Discussion

Summary of Research

We took a molecular approach aimed to find regulatory elements that control the expression of APP. APP is known to contain cis-regulatory elements within its mRNA sequence that regulates its protein levels. Furthermore, mutations in the APP gene leads to early onset AD. Our lab identified a guanine rich region in the 3'UTR of APP that folds into a guanine quadruplex structure, which negatively regulates its translation [113]. Guanine rich RNA can adopt a secondary structure known as a guanine quadruplex (G-quadruplex). Formation of the G-quadruplex occurs through Hoosteen hydrogen bonding between the guanine bases and are stabilized by monovalent cations such as potassium or sodium [151]. It is predicted to be as many as 376,000 G-quadruplex forming sequences in the human genome [156]. RNA G-quadruplexes are located in 5'UTRs, coding sequences, and 3'UTRs where they regulate mRNA localization [162] and predominantly suppress mRNA translation [115, 174-176, 234].

The G-quadruplex was identified in the 3'UTR of APP is highly conserved, suggesting that it may have an important biological function (**Figure 2.1**). We used circular dichroism (CD) to investigate the structural formation (**Figure 2.2**) and further tested the formation of this secondary structure was potassium dependent (**Figure 2.2**). The G-quadruplex was stabilized by K+ at physiological concentration, suggesting that this structure could fold within cells. Furthermore, mutating the G-quadruplex altered its stability, by preventing folding of this sequence (**Figure 2.2**).

We next investigated whether the G-quadruplex sequence had any biological effect on APP levels [113]. We used a combination of reporter constructs containing the intact or the mutated G-quadruplex sequence (**Figure 2.3** and **Figure 2.4**) Our results indicated that the intact APP 3'UTR G-quadruplex sequence negatively regulates reporter gene expression as well APP protein steady state levels and APP translation (**Figure 2.4** and **Figure 2.5).** Moreover, mutations in the APP 3'UTR G-quadruplex sequence lead to higher expression of APP levels and an increase in Aβ levels (**Figure 2.4**). These results indicate, for the first time, that APP contains a 3'UTR G-quadruplex and that its expression is regulated by this sequence. Since over-expression of APP protein levels can lead to increased Aβ production, further understanding into the mechanism of how the G-quadruplex regulates APP is warranted.

We decided to continue looking into the mechanism by which APP expression is regulated by its 3'UTR G-quadruplex as a follow up from our published manuscript. There are numerous reports investigating post-transcriptional of gene expression by RNA G-quadruplexes. The major findings are that these structures serve a binding sites for RNA binding proteins that facilitate translation control of gene expression. We decided to investigate identify proteins that bind to this G-quadruplex sequence in order to further characterize how the G-quadruplex mediates translational regulation of APP. Summarized here are our unpublished findings and preliminary results findings in which we identified 78 putative RNA binding proteins from our mass spectrometry analysis that favor the binding to the intact APP 3'UTR G-quadruplex sequence (**Table 4.2**). Of those 78 putative proteins, FMRP and FXR2P were selected for further investigation. Our results indicate, for the first time, that both FMRP and FXR2P bind to the APP 3'UTR G-

quadruplex sequence (**Figure 4.3**). Our western blot analysis reveals that the binding of these proteins is stronger for the intact APP 3'UTR, and binds less favorably to the mutant sequence. This data suggests that the binding of FMRP and FXR2P is dependent of the folding of the APP 3'UTR G-quadruplex. Furthermore, we report for the first time that FXR2P over-expression reduces APP levels (**Figure 4.4**). Lastly, we report preliminary data, which for the first time, indicates that FMRP and FXR2P reduce APP expression mediated by the 3'UTR G-quadruplex; however, additional experiments are needed to confirm this finding.

Future Directions

Determine How FMRP and FXR2P Regulates APP Expression via G-quadruplex

FMRP and FXR2P contain conserved RNA binding domains such as two KH domains and the RGG box. While FXR2P has not been shown to bind G-quadruplex sequence, FMRP has been shown to bind to such sequences through its RGG box domain [248, 264, 265]. Addressing whether FMRP, or FXR2P, uses its RGG box domain to bind to the APP 3'UTR G-quadruplex in order to regulate APP expression warrants further investigation. Additionally, it was reported that FMRP binds to the coding sequence of APP mRNA [96] and we cannot definitively rule out whether the guanine rich region in the coding sequence of APP alone is necessary for FMRP to regulation APP expression. Our preliminary data suggests that FMRP regulates APP expression through the 3'UTR G-quadruplex, which may suggest that FMRP may selectively or preferentially use the G-quadruplex sequence to regulate APP expression, however, further experiments are needed to draw this conclusion.

In fmr1 KO mice, high levels of APP and Aβ have been reported [96]. Both FMRP and FXR2P seem to have the ability to reduce APP levels; however, it remains to be determined if they regulate APP levels cooperatively or independent of one another. In fragile X syndrome, FXR2P cannot completely compensate for the lack of FMRP, which suggests that FMRP and FXR2P work together to regulate translation of mRNAs. While FMRP is known to suppress APP translation, FXR2P has not previously been shown to interact with APP or regulate its expression.

Identify Additional Proteins that Bind APP 3'UTR G-quadruplex

Our mass spectrometry results yielded several proteins that could interact with the intact APP 3'UTR G-quadruplex sequence (**Table 4.2**). It's important to note that we only used the APP 3'UTR G-quadruplex sequence and the proteins identified may not reflect endogenous proteins that bind to the APP mRNA. Several proteins are known to bind G-quadruplex structures [186] and it's quite possible that the proteins which bind the APP 3'UTR G-quadruplex, may not bind under endogenous conditions, but bind to the sequence only. One way to circumvent this caveat would be to use the protein which binds the G-quadruplex sequence and use that protein to pull down the APP mRNA from cell lysate. If identified protein is capable of pulling down the APP mRNA, we can then determine if the interaction is G-quadruplex dependent. Further experiments are needed to determine if there are additional proteins that bind to this sequence and explore additional mechanisms of regulation. Several proteins from our mass spectrometry analysis bind to G-quadruplex in other mRNAs such as nucleolin [235, 276], which destabilizes APP mRNA and suppress its translation [101]; Fus [277], and G3BP2 [193] . G-quadruplex sequences can target the initiation step in translation. One possible way

this can be achieved is by proteins binding to the sequence and thus interfere with eukaryotic initiation factors in order to suppress translation or by sequestering these initiation factors to promote translation by cap-independent translation. Our results indicate that further exploration is needed to fully characterize how the APP 3'UTR G-quadruplex mediates translational control of APP expression and may provide additional insight into the regulation of APP and how it is involved in AD pathogenesis.

Identify Mutations or Single Nucleotide Polymorphisms in Endogenous APP mRNA Regulatory Elements

mRNAs serve as the substrate for translation. Within the mRNA are cis regulatory elements which bind trans regulatory elements that facilitate translational control [212]. Therefore, mutations found within the cis regulatory elements could alter the binding of trans acting factors and mutations in trans acting factors could alter how they bind to the cis regulatory element and thus affect translation. We identified a new cis regulatory element known as the G-quadruplex in the 3'UTR of APP. The G-quadruplex negatively regulates APP translation [113]. We show preliminary data indicating FMRP and FXR2P binds to the G-quadruplex sequence and regulates APP translation. Future experiments will investigate whether mutations or single nucleotide polymorphisms (SNPs) exist in the APP 3'UTR G-quadruplex sequence. We hypothesize that mutations or SNPs could prevent G-quadruplex from properly forming and could increase APP levels. This was demonstrated by the mutations our lab produced in the APP 3'UTR G-quadruplex sequence and that those mutations prevented the folding of the G-quadruplex sequence and increased APP levels. Moreover, we identified proteins that bound to the intact G-quadruplex sequence (unpublished data, Table 4.2). We would next investigate whether

mutations are found in those proteins, and further investigate whether those mutations alter their binding to the APP 3'UTR G-quadruplex and the consequence it has on regulating APP levels.

Discussion

G-quadruplex structure and function: From K^+ stacking to K^+ sensors?

The work summarized in this thesis examined how the presence or absence of the G-quadruplex regulated APP translation. Upon the observations in figure 2.2C, we noted a potassium mediated stacking of the APP 3'UTR G-quadruplex sequence. At intracellular potassium levels, this sequence would adopt a three-stacked G-quadruplex. However, we did not include the intermediate two-stacked G-quadruplex sequence, which would occur at a lower potassium concentration. Two-stacked G-quadruplex structures have been experimentally shown to be weaker and less stabilized structures when compared to G-quadruplexes with more tetrad stacks [278]. However, two-stacked G-quadruplex structures are capable of regulating translation of mRNAs that harbor these structures [278, 279]. It's not surprising that potassium mediates the stacking of the APP 3'UTR G-quadruplex sequence. It can lead one to speculate that changes in potassium concentration could cause a switch between a three-stacked to a two-stacked G-quadruplex structure, and could possibly affect translational regulation for APP, since we previously showed that weakening the G-quadruplex sequence leads to increased APP levels. Potassium is the major intracellular ion whose concentration is higher inside of the cell [280]. The availability of potassium intracellularly could be a reason why this ion is preferred to stabilize G-quadruplex structures. It well known that potassium stabilizes G-

quadruplex due to the fact that potassium has a larger atomic radius and can interact with two quartets within a G-quadruplex structure [281]. Potassium channels therefore could potentially influence G-quadruplex formation by modulating intracellular potassium levels. Upon activation, potassium channels mediate the efflux of K^+ ions [282]. Given that G-quadruplex stacking is dependent on potassium concentration, it would be interesting to determine if modulation of K^+ could affect G-quadruplex stacking, and moreover, could have an effect on translation. Modulating potassium concentrations could provide a mechanism to induce localized translation of mRNAs through changes in intracellular K^+ concentration by altering the stacking of the G-tetrads; therefore, G-quadruplex structures could be used as a switch in response to K^+ concentration and modulate translation. For instance, FMRP has recently been shown to have a function outside of regulating mRNA translation in that FMRP can regulate the activation of potassium channels. In particular, it was shown that FMRP interacts with the β4 subunit of BK channels [283], which are especially sensitive to calcium depolarization [284]. Binding to FMRP to the β4 subunit of BK channels promotes potassium efflux, which is able to increase membrane repolarization [283]. Could FMRP's role in activating BK channels have an effect on the translation of FMRP target mRNAs that harbor G-quadruplex structures stabilized by potassium concentration? Further exploration of this idea is warranted as this could provide a mechanism of localized translation.

The challenge to investigate this idea is performing the appropriate experiment in cells. The vast majority of G-quadruplex studies have been investigated outside of cells. It has been experimentally validated that DNA G-quadruplexes could act as sensors to detect changes in potassium concentration [285]. A promising technique that was developed by

Liu and colleagues [286], utilized bimolecular fluorescence complementation which enabled them to study the formation of G-quadruplex structures under intracellular conditions in living cells. In their study, they used a RNA sequence which contained a G-quadruplex sequence that was in close proximity to an aptamer tag. Additionally, a split eGFP protein was fused a protein bound which bound to the aptamer sequence as well as to the G-quadruplex sequence. Once inside the cell, the split eGFP tagged proteins were able to bind either to the aptamer or to the fully folded G-quadruplex sequence. Once the tagged proteins were able to bind to the sequences, it enabled the detection of the eGFP [286]. Mutations that prevented the formation of the G-quadruplex as well as lithium ion (which do not stabilize G-quadruplex structures) precluded the interaction of the split eGFP fusion proteins and therefore was not detected [286]. This technique could be of interest to study how G-quadruplex structures change in response to intracellular potassium concentrations and could provide insight into how those changes affect translation of mRNAs that harbor such structures.

G-quadruplexes and miRNAs: Synergistic Regulators of translation?

G-quadruplexes are located near miRNA binding sequences and proteins that bind to this structure can interact with the miRNA pathway [235, 287]. FMRP and Nucleolin have been shown to interact with AGO2 protein in the miRISC complex as well as G-quadruplexes [189, 235, 287]. This data suggests that these proteins can participate in translational regulation with miRNAs. Several miRNAs repress APP translation through the 3’UTR [106, 231]. Of the miRNAs previously identified, one family of miRNAs, which includes has-mir-106a, 106b, and 17, are in close proximity to the newly identified

G-quadruplex sequence (**Figure 5.1**). These miRNAs are highly conserved and share a common miRNA binding sequence. We wanted to test whether miRNA regulations could be influenced by the presence or absence of the G-quadruplex sequence in APP. Using a luciferase reporter construct, we sub cloned the entire APP 3'UTR with either the wild type or mutant G-quadruplex sequence. Since miRNAs bind to their complementary sequences of its target mRNAs, we did not mutate the regions corresponding to their miRNA binding sites. If miRNAs regulate luciferase gene expression independent of the G-quadruplex, we would expect to see a decrease in luciferase expression in the presence or absence of the G-quadruplex. We used hsa-mir 106a and 153 for this experiments. hsa-mir 153 is located ~ 220 nucleotides upstream of the has-mir 106a binding sequence. Both hsa-mir 106a and 153 negatively regulates APP expression through binding in the APP 3'UTR. Preliminary data in **Figure 5.2** suggests that the APP 3'UTR G-quadruplex may influence miRNA mediated regulation of gene expression. However, additional experiments are needed.

Since we did not mutate the miRNA binding sequences, the miRNAs should be able to bind and regulate gene expression; however, that is not case when we mutate the G-quadruplex. One can speculate that other regulatory elements, RNA binding proteins perhaps, may be needed to bind the G-quadruplex structure in order to recruit miRNA machinery for translational repression. As the 3'UTR of APP contains several cis/trans regulatory elements, further exploration for how this factors influence APP translation is warranted.

APP 3'UTR G-quadruplex Sequence is common to PPP2CB

We blasted the APP 3'UTR G-quadruplex sequence to determine how prevalent this sequence is in the human genome (**Figure 5.3**). The results indicate that the APP 3'UTR G-quadruplex sequence is very unique to the APP mRNA. One transcript, protein phosphatase 2 catalytic subunit beta (PPP2CB), was found to share the complete APP 3'UTR G-quadruplex sequence in its 5'UTR. PPP2CB is the catalytic subunit of the protein phosphatase 2A. Protein phosphatase 2A plays a role in Alzheimer's disease as downregulation of this proteins leads to tau hyperphosphorylation, which results in neurofibrillary tangles observed in AD brains [288]. As this sequence is known to form a G-quadruplex structure and regulate APP expression, there is opportunities to explore how this sequence regulates PPP2CB and how that affects the function of protein phosphatase 2A function. The

APP, ADAM-10, and BACE contain G-quadruplex Sequence

While the G-quadruplex sequence regulates APP expression, this secondary structure is also found in both β- and α- secretases. Both β- and α- secretases are the initial cleavage enzymes that lead to the amyloidogenic (β-secretase) or non-amyloidogenic (α-secretase) pathways [137]. The gene that encodes β-secretase is BACE1. BACE1 has a G-quadruplex sequence in the located in exon 3 region which regulates its alternative splicing [289]. This G-quadruplex sequence leads to the proteolytically active full length BACE1, while mutations in this sequence give produces the inactive BACE1 transcript [289]. Additionally, ADAM-10, the gene that encodes α-secretase, has a G-quadruplex in its 5'UTR that that represses its translation [290]. Mutations in this G-quadruplex sequence results in increased cleavage of APP by α-secretase [290]. As APP, BACE1, and ADAM-10 expression can be regulated by G-quadruplex sequences, it will be

important to further investigate how this structure regulates the expression of these genes that play such pivotal role in AD.

Concluding Remarks:

Alzheimer's disease (AD), the most common form of dementia, is a devastating neurological disease which results in synaptic dysfunction and neuronal loss. The accumulation senile plaques and neurofibrillary tangles are molecular hallmarks in AD pathogenesis, which arise from amyloid-β (Aβ) peptides and hyperphosphorylated tau protein respectively. Aβ deposition is at the core of the amyloid cascade hypothesis which postulates that elevated Aβ levels is the central event that leads to AD pathology [291]. Aβ peptides are produced by sequential, proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ- secretases [137]. The biological function of APP is not well understood; however, APP can modulate neurite outgrowth and neuronal migration and has also been shown to be involved in synapse formation and maintenance [46].

Indeed, proteolytic cleavage of APP leads to Aβ production and this effect is exacerbated due to over-expression of APP protein levels which leads to an increase in the production of Aβ. APP is located on chromosome 21 and research indicates that individuals with trisomy 21 (Down Syndrome (DS)) are at a high risk of developing AD [197, 198]. Several studies suggest there is a dose-dependent increase of APP in individuals with DS, as those individuals have an increase in APP levels which corresponds to an increase in Aβ levels [199]. Interestingly, individuals with DS develop an early onset of AD starting with evidence of neuropathological hall marks of AD, Aβ plaques and neurofibrillary

tangles, around age 40 [9, 200]. In addition to over expression of APP due to trisomy 21, the Swedish mutation found in APP causes an increase in APP levels and increases the cleavage of APP by beta and gamma secretases, thereby releasing more Aβ [201, 202].

While there is no cure for AD, the main strategies for therapeutic development are aimed at decreasing Aβ levels by targeting the enzymes that mediate its generation and to increase Aβ clearance [29, 30, 292]. Another strategy to find drug targets for AD, based on the amyloid hypothesis, involves identifying proteins or molecules that regulate APP protein levels. Given that over expression of APP contributes to AD pathology, it will be necessary to explore regulatory elements that could modulate APP levels.

With the identification of the G-quadruplex sequence in the 3'UTR of APP and further demonstrating this sequence negatively regulates APP expression, there are additional opportunities for research aimed at investigating the mechanism of regulation by this sequence. By exploring proteins and additional regulatory elements that interact with the G-quadruplex sequence, it may provide further insight in our understanding of Alzheimer's disease.

Figure Legends

Figure 5.1: G-quadruplex is located near miRNA binding sites Schematic of APP 3'UTR sequence for miRNA binding and G-quadruplex. The red nucleotides correspond to the binding of miRNA family members 106a, 106b, and 17 in position 2980-3005 of the APP 3'UTR. G-quadruplex sequence starts just 3 nucleotides from the ending of the miRNA binding sequence (position 3008-3027).

Figure 5.2: Preliminary Data: G-quadruplex alters miRNA function: Quantification of dual luciferase assay from cells over-expressing miRNAs 106a and 153 with luciferase reporter constructs containing wild type (WT) or mutant (Mut) G-quadruplex sequence. Over-expression of miRNAs with the luciferase reporter (WT G-quadruplex) construct shows a decrease in luciferase levels. Conversely, over-expression of miRNAs with the mutant G-quadruplex luciferase construct failed to reduce luciferase levels. Y-axis is the amount of firefly luciferase gene expression normalized to renilla luciferase levels.

Figure 5.3: Nucleotide Blast of APP 3'UTR G-quadruplex Sequence

Figure 5.1

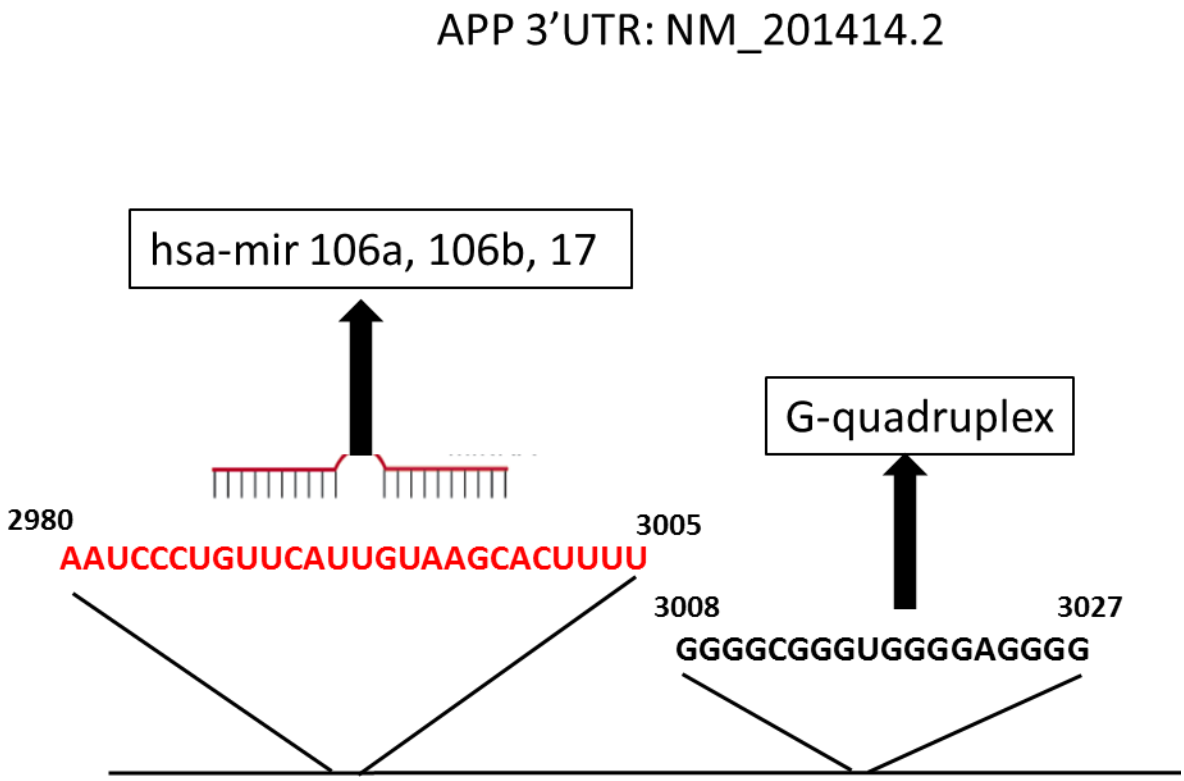

APP 3'UTR: NM_201414.2
hsa-mir 106a, 106b, 17
G-quadruplex
2980
3005
AAUCCCUGUUCAUUGUAAGCACUUUU
3008
3027
GGGGCGGGUGGGGAGGGG

Figure 5.2

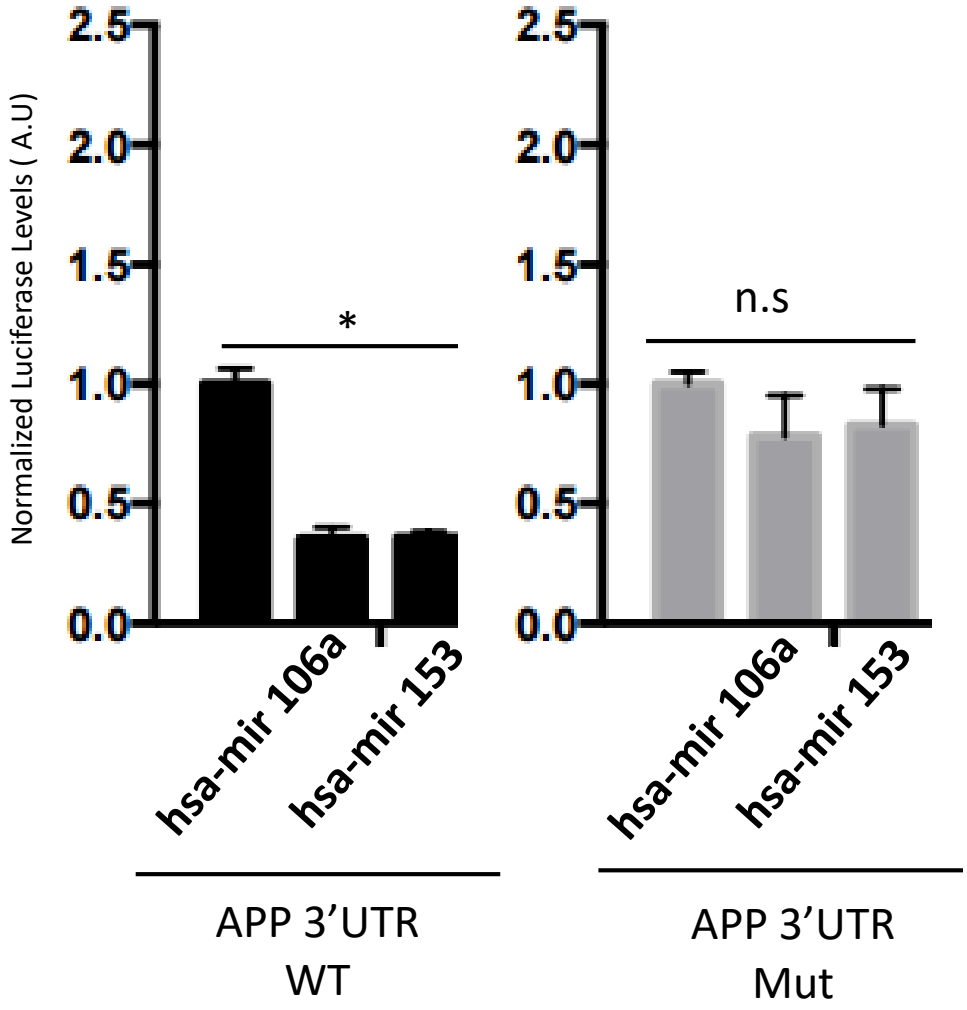
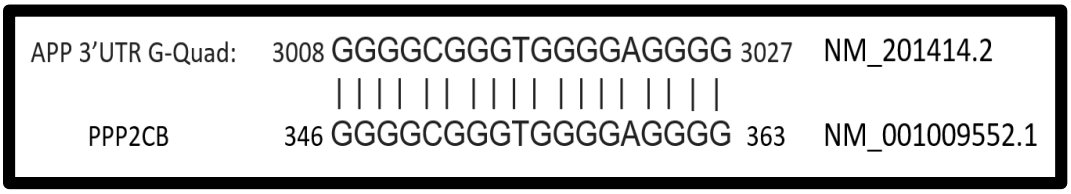

Normalized Luciferase Levels (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
*
n.s
hsa-mir 106a
hsa-mir 153
hsa-mir 106a
hsa-mir 153
APP 3'UTR
WT
APP 3'UTR
Mut

Figure 5.3


APP 3'UTR G-Quad: 3008 GGGGCGGGTGGGGAGGGG 3027 NM_201414.2
PPP2CB 346 GGGGCGGGTGGGGAGGGG 363 NM_001009552.1

References

1. Katzman, R., *Maintenance of a constant brain extracellular potassium.* Fed Proc, 1976. **35**(6): p. 1244-7.
2. Tanzi, R.E. and L. Bertram, *Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective.* Cell, 2005. **120**(4): p. 545-555.
3. Tarawneh, R. and D.M. Holtzman, *The clinical problem of symptomatic Alzheimer disease and mild cognitive impairment.* Cold Spring Harbor perspectives in medicine, 2012. **2**(5): p. a006148.
4. Bird, T.D., *Genetic aspects of Alzheimer disease.* Genetics in medicine : official journal of the American College of Medical Genetics, 2008. **10**(4): p. 231-239.
5. *2016 Alzheimer's disease facts and figures.* Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 2016. **12**(4): p. 459-509.
6. Bekris, L.M., et al., *Genetics of Alzheimer disease.* Journal of geriatric psychiatry and neurology, 2010. **23**(4): p. 213.
7. Goldgaber, D., et al., *Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease.* Science, 1987. **235**(4791): p. 877-880.
8. Tanzi, R.E., et al., *Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus.* Science, 1987. **235**(4791): p. 880-884.
9. Raux, G., et al., *APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy.* Nature Genetics, 2006. **38**(1): p. 24-26.
10. Sherrington, R., et al., *CLONING OF A GENE BEARING MISSENSE MUTATIONS IN EARLY-ONSET FAMILIAL ALZHEIMERS-DISEASE.* NATURE, 1995. **375**(6534): p. 754-760.
11. Rogaev, E.I., et al., *FAMILIAL ALZHEIMERS-DISEASE IN KINDREDS WITH MISSENSE MUTATIONS IN A GENE ON CHROMOSOME-1 RELATED TO THE ALZHEIMERS-DISEASE TYPE-3 GENE.* NATURE, 1995. **376**(6543): p. 775-778.
12. Koedam, E., et al., *Early-Versus Late-Onset Alzheimer's Disease: More than Age Alone.* JOURNAL OF ALZHEIMERS DISEASE, 2010. **19**(4): p. 1401-1408.
13. Isik, A.T., *Late onset Alzheimer's disease in older people.* CLINICAL INTERVENTIONS IN AGING, 2010. **5**: p. 307-311.
14. Van Cauwenberghe, C., C. Van Broeckhoven, and K. Sleegers, *The genetic landscape of Alzheimer disease: clinical implications and perspectives.* Genetics in medicine : official journal of the American College of Medical Genetics 2016. **18**(5): p. 421.
15. Corder, E.H., et al., *Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families.* Science (New York, N.Y.), 1993. **261**(5123): p. 921-923.
16. Bertram, L. and R.E. Tanzi, *Genome-wide association studies in Alzheimer's disease.* HUMAN MOLECULAR GENETICS, 2009. **18**(R2): p. R137-R145.
17. Zeller, J.L., *Conjugal Alzheimer Disease: Risk in Children When Both Parents Have Alzheimer Disease.* JAMA, 2008. **299**(19): p. 2259.
18. Ngandu, T., et al., *A 2 year multidomain intervention of diet, exercise, cognitive training, and vascular risk monitoring versus control to prevent cognitive decline in at-risk elderly people (FINGER): a randomised controlled trial.* LANCET, 2015. **385**(9984): p. 2255-2263.
19. West, M.J., et al., *Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease.* Lancet, 1994. **344**(8925): p. 769-72.
20. Shankar, G.M. and D.M. Walsh, *Alzheimer's disease: synaptic dysfunction and A beta.* MOLECULAR NEURODEGENERATION, 2009. **4**(1): p. 48-48.

21. Nixon, R.A., *Autophagy, amyloidogenesis and Alzheimer disease.* JOURNAL OF CELL SCIENCE, 2007. **120**(23): p. 4081-4091.
22. Crews, L. and E. Masliah, *Molecular mechanisms of neurodegeneration in Alzheimer's disease.* HUMAN MOLECULAR GENETICS, 2010. **19**(R1): p. R12-R20.
23. Ballatore, C., V.M.Y. Lee, and J.Q. Trojanowski, *Tau-mediated neurodegeneration in Alzheimer's disease and related disorders.* NATURE REVIEWS NEUROSCIENCE, 2007. **8**(9): p. 663-672.
24. Morishima-Kawashima, M. and Y. Ihara, *Alzheimer's disease: β-Amyloid protein and tau.* Journal of Neuroscience Research, 2002. **70**(3): p. 392-401.
25. Spires-Jones, T.L. and B.T. Hyman, *The Intersection of Amyloid Beta and Tau at Synapses in Alzheimer's Disease.* NEURON, 2014. **82**(4): p. 756-771.
26. Jin, M., et al., *Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration.* Proc Natl Acad Sci U S A, 2011. **108**(14): p. 5819-24.
27. Selkoe, D.J. and J. Hardy, *The amyloid hypothesis of Alzheimer's disease at 25 years.* EMBO molecular medicine, 2016. **8**(6): p. 595-608.
28. Karran, E., M. Mercken, and B. De Strooper, *The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics.* NATURE REVIEWS DRUG DISCOVERY, 2011. **10**(9): p. 698-U1600.
29. De Strooper, B. and W. Annaert, *Proteolytic processing and cell biological functions of the amyloid precursor protein.* Journal of cell science, 2000. **113 (Pt 11)**(Pt 11): p. 1857-1870.
30. Vassar, R., et al., *Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE.* Science (New York, N.Y.), 1999. **286**(5440): p. 735-741.
31. MacLeod, R., et al., *The role and therapeutic targeting of α-, β- and γ-secretase in Alzheimer's disease.* Future Science OA, 2015. **1**(3).
32. Hu, X., et al., *Bace1 modulates myelination in the central and peripheral nervous system.* Nature neuroscience, 2006. **9**(12): p. 1520-1525.
33. Wong, H.-K., et al., *beta Subunits of voltage-gated sodium channels are novel substrates of beta-site amyloid precursor protein-cleaving enzyme (BACE1) and gamma-secretase.* The Journal of biological chemistry, 2005. **280**(24): p. 23009.
34. Dominguez, D., et al., *Phenotypic and Biochemical Analyses of BACE1- and BACE2-deficient Mice.* Journal of Biological Chemistry, 2005. **280**(35): p. 30797-30806.
35. De Strooper, B., et al., *Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein.* Nature, 1998. **391**(6665): p. 387-390.
36. Augelli-Szafran, C.E., et al., *Discovery of notch-sparing gamma-secretase inhibitors.* Current Alzheimer research U6 - externalDocID=20088802&paramdict=en-US U7 - Journal Article, 2010. **7**(3): p. 207.
37. Golde, T.E., et al., *γ-Secretase inhibitors and modulators.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2013. **1828**(12): p. 2898-2907.
38. Coric, V., et al., *Safety and Tolerability of the γ-Secretase Inhibitor Avagacestat in a Phase 2 Study of Mild to Moderate Alzheimer Disease.* Archives of Neurology, 2012. **69**(11): p. 1430-1440.
39. Castello, M.A. and S. Soriano, *Rational heterodoxy: cholesterol reformation of the amyloid doctrine.* Ageing research reviews, 2013. **12**(1): p. 282-288.

40. Drachman, D.A., *The amyloid hypothesis, time to move on: Amyloid is the downstream result, not cause, of Alzheimer's disease.* Alzheimer's & dementia : the journal of the Alzheimer's Association, 2014. **10**(3): p. 372-380.
41. Folch, J., et al., *Current Research Therapeutic Strategies for Alzheimer's Disease Treatment.* Neural plasticity, 2016. **2016**: p. 8501693.
42. Zhang, Y.W., et al., *APP processing in Alzheimer's disease.* Molecular brain, 2011. **4**: p. 3-6606-4-3.
43. Zheng, H. and E.H. Koo, *The amyloid precursor protein: beyond amyloid.* Molecular neurodegeneration, 2006. **1**: p. 5.
44. O'Brien, R.J. and P.C. Wong, *Amyloid precursor protein processing and Alzheimer's disease.* Annual review of neuroscience, 2011. **34**(1): p. 185-204.
45. Haass, C., et al., *Trafficking and proteolytic processing of APP.* Cold Spring Harbor perspectives in medicine, 2012. **2**(5): p. a006270.
46. Hoe, H.S., H.K. Lee, and D.T. Pak, *The upside of APP at synapses.* CNS neuroscience & therapeutics, 2012. **18**(1): p. 47-56.
47. Zheng, H., et al., *beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity.* Cell, 1995. **81**(4): p. 525-531.
48. Dawson, G.R., et al., *Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein.* Neuroscience, 1999. **90**(1): p. 1-13.
49. Simón, A.-M., et al., *Overexpression of wild-type human APP in mice causes cognitive deficits and pathological features unrelated to Aß levels.* Neurobiology of Disease, 2009. **33**(3): p. 369-378.
50. Dawkins, E. and D.H. Small, *Insights into the physiological function of the beta-amyloid precursor protein: beyond Alzheimer's disease.* J Neurochem, 2014. **129**(5): p. 756-69.
51. Selkoe, D. and R. Kopan, *Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration.* Annual Review of Neuroscience, 2003. **26**: p. 565-597.
52. Lorenzo, A., et al., *Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease.* NATURE NEUROSCIENCE, 2000. **3**(5): p. 460-464.
53. Thinakaran, G. and E.H. Koo, *Amyloid precursor protein trafficking, processing, and function.* J Biol Chem, 2008. **283**(44): p. 29615-9.
54. Wang, Z., et al., *Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2009. **29**(35): p. 10788-10801.
55. Young-Pearse, T.L., et al., *A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2007. **27**(52): p. 14459-14469.
56. Young-Pearse, T.L., et al., *Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1.* NEURAL DEVELOPMENT, 2008. **3**(1): p. 15-15.
57. Hébert, S.S., et al., *Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes.* EMBO reports, 2006. **7**(7): p. 739-745.
58. Lichtenthaler, S.F., C. Haass, and H. Steiner, *Regulated intramembrane proteolysis – lessons from amyloid precursor protein processing.* Journal of Neurochemistry, 2011. **117**(5): p. 779-796.

59. Tian, Y., et al., *An APP inhibitory domain containing the Flemish mutation residue modulates γ-secretase activity for Aβ production.* Nature Structural & Molecular Biology, 2010. **17**(2): p. 151-158.
60. Mattson, M.P., et al., *Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the β-amyloid precursor protein.* Neuron, 1993. **10**(2): p. 243-254.
61. Barger, S.W., et al., *Activation of K + channels and suppression of neuronal activity by secreted β-amyloid-precursor protein.* Nature, 1996. **379**(6560): p. 74-78.
62. Gakhar-Koppole, N., et al., *Activity requires soluble amyloid precursor protein alpha to promote neurite outgrowth in neural stem cell-derived neurons via activation of the MAPK pathway.* The European journal of neuroscience, 2008. **28**(5): p. 871-882.
63. O'Leary, D.D.M., et al., *APP binds DR6 to trigger axon pruning and neuron death via distinct caspases.* Nature, 2009. **457**(7232): p. 981-989.
64. Kamenetz, F., et al., *APP Processing and Synaptic Function.* Neuron, 2003. **37**(6): p. 925-937.
65. von Rotz, R.C., et al., *The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor.* Journal of cell science, 2004. **117**(Pt 19): p. 4435-4448.
66. Cao, X. and T.C. Südhof, *A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60.* Science (New York, N.Y.), 2001. **293**(5527): p. 115.
67. Ozaki, T., et al., *The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis.* Biochemical and Biophysical Research Communications, 2006. **351**(1): p. 57-63.
68. S.J, T., et al., *alpha- and beta-secretase: profound changes in Alzheimer's disease.* Biochemical and Biophysical Research Communications, 2002. **299**(3): p. 373-376.
69. Giaccone, G., et al., *Down patients: Extracellular preamyloid deposits precede neuritic degeneration and senile plaques.* Neuroscience Letters, 1989. **97**(1): p. 232-238.
70. Lemere, C.A., et al., *Sequence of Deposition of Heterogeneous Amyloid β-Peptides and APO E in Down Syndrome: Implications for Initial Events in Amyloid Plaque Formation.* Neurobiology of Disease, 1996. **3**(1): p. 16-32.
71. Haass, C., et al., *The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway.* Nat Med, 1995. **1**(12): p. 1291-6.
72. Wang, X., M.L. Michaelis, and E.K. Michaelis, *Functional Genomics of Brain Aging and Alzheimers Disease: Focus on Selective Neuronal Vulnerability.* Current Genomics, 2010. **11**(8): p. 618-633.
73. Lv, H., J. Jia, and L. Jia, *Promoter polymorphisms which modulate APP expression may increase susceptibility to Alzheimer's disease.* Neurobiology of Aging, 2008. **29**(2): p. 194-202.
74. Lahiri, D.K., *MOLECULAR ANALYSIS OF THE PROMOTER REGION OF THE GENE ENCODING THE BETA-AMYLOID PRECURSOR PROTEIN.* INDIAN JOURNAL OF BIOCHEMISTRY & BIOPHYSICS, 1995. **32**(6): p. 329-335.
75. Vostrov, A.A. and W.W. Quitschke, *The zinc finger protein CTCF binds to the APBbeta domain of the amyloid beta-protein precursor promoter. Evidence for a role in transcriptional activation.* The Journal of biological chemistry, 1997. **272**(52): p. 33353-33359.

76. Dewji, N.N. and C. Do, *Heat shock factor-1 mediates the transcriptional activation of Alzheimer's β-amyloid precursor protein gene in response to stress.* Molecular Brain Research, 1996. **35**(1): p. 325-328.
77. Grilli, M., et al., *IDENTIFICATION AND CHARACTERIZATION OF A KAPPA-B/REL BINDING-SITE IN THE REGULATORY REGION OF THE AMYLOID PRECURSOR PROTEIN GENE.* JOURNAL OF BIOLOGICAL CHEMISTRY, 1995. **270**(45): p. 26774-26777.
78. Beyreuther, K., et al., *REGULATION AND EXPRESSION OF THE ALZHEIMERS BETA/A4 AMYLOID PROTEIN-PRECURSOR IN HEALTH, DISEASE, AND DOWNS-SYNDROME*. 1993, NEW YORK ACAD SCIENCES: NEW YORK. p. 91-102.
79. Pollwein, P., C.L. Masters, and K. Beyreuther, *The expression of the amyloid precursor protein (APP) is regulated by two GC-elements in the promoter.* Nucleic Acids Research, 1992. **20**(1): p. 63-68.
80. Brouwers, N., et al., *Genetic risk and transcriptional variability of amyloid precursor protein in Alzheimer's disease.* BRAIN, 2006. **129**(11): p. 2984-2991.
81. Goldgaber, D., et al., *Interleukin 1 Regulates Synthesis of Amyloid β -protein Precursor mRNA in Human Endothelial Cells.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(19): p. 7606-7610.
82. Dinarello, C.A., *Biologic basis for interleukin-1 in disease.* BLOOD, 1996. **87**(6): p. 2095-2147.
83. Van den Heuvel, C., et al., *Upregulation of Amyloid Precursor Protein Messenger RNA in Response to Traumatic Brain Injury: An Ovine Head Impact Model.* Experimental Neurology, 1999. **159**(2): p. 441-450.
84. Plummer, S., et al., *The Neuroprotective Properties of the Amyloid Precursor Protein Following Traumatic Brain Injury.* AGING AND DISEASE, 2016. **7**(2): p. 163-179.
85. Day, D.A. and M.F. Tuite, *Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview.* JOURNAL OF ENDOCRINOLOGY, 1998. **157**(3): p. 361-371.
86. Beaudoin, M.E., V.-J. Poirel, and L.A. Krushel, *Regulating amyloid precursor protein synthesis through an internal ribosomal entry site.* Nucleic Acids Research, 2008. **36**(21): p. 6835-6847.
87. Rogers, J.T., et al., *Translation of the Alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5 '-untranslated region sequences.* JOURNAL OF BIOLOGICAL CHEMISTRY, 1999. **274**(10): p. 6421-6431.
88. Rogers, J.T., et al., *An iron-responsive element type II in the 5 '-untranslated region of the Alzheimer's amyloid precursor protein transcript.* JOURNAL OF BIOLOGICAL CHEMISTRY, 2002. **277**(47): p. 45518-45528.
89. Cho, H.H., et al., *Selective Translational Control of the Alzheimer Amyloid Precursor Protein Transcript by Iron Regulatory Protein-1.* JOURNAL OF BIOLOGICAL CHEMISTRY, 2010. **285**(41): p. 31217-31232.
90. Westmark, C.J. and J.S. Malter, *The regulation of AbetaPP expression by RNA-binding proteins.* Ageing research reviews, 2012. **11**(4): p. 450-459.
91. Darnell, J.C., et al., *Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function.* Cell, 2001. **107**(4): p. 489-499.
92. Schaeffer, C., et al., *The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif.* The EMBO Journal, 2001. **20**(17): p. 4803-4813.
93. Das, I., et al., *Hedgehog agonist therapy corrects structural and cognitive deficits in a Down syndrome mouse model.* Science translational medicine, 2013. **5**(201): p. 201ra120.

94. Darnell, J.C., et al., *FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism.* Cell, 2011. **146**(2): p. 247-261.
95. Ascano, M., Jr., et al., *FMRP targets distinct mRNA sequence elements to regulate protein expression.* Nature, 2012. **492**(7429): p. 382-386.
96. Westmark, C.J. and J.S. Malter, *FMRP mediates mGluR5-dependent translation of amyloid precursor protein.* PLoS biology, 2007. **5**(3): p. e52.
97. Westmark, C.J., P.R. Westmark, and J.S. Malter, *MPEP reduces seizure severity in Fmr-1 KO mice over expressing human Abeta.* International journal of clinical and experimental pathology, 2009. **3**(1): p. 56-68.
98. Lee, E.K., et al., *hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies.* Nature structural & molecular biology, 2010. **17**(6): p. 732-739.
99. Chaudhury, A., P. Chander, and P.H. Howe, *Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles.* RNA (New York, N.Y.), 2010. **16**(8): p. 1449-1462.
100. Rajagopalan, L.E., et al., *hnRNP C increases amyloid precursor protein (APP) production by stabilizing APP mRNA.* NUCLEIC ACIDS RESEARCH, 1998. **26**(14): p. 3418-3423.
101. Zaidi, S.H. and J.S. Malter, *Nucleolin and heterogeneous nuclear ribonucleoprotein C proteins specifically interact with the 3'-untranslated region of amyloid protein precursor mRNA.* The Journal of biological chemistry, 1995. **270**(29): p. 17292-17298.
102. Broytman, O., et al., *Rck/p54 interacts with APP mRNA as part of a multi-protein complex and enhances APP mRNA and protein expression in neuronal cell lines.* Neurobiology of Aging, 2009. **30**(12): p. 1962-1974.
103. Doench, J.G. and P.A. Sharp, *Specificity of microRNA target selection in translational repression.* Genes & development, 2004. **18**(5): p. 504-511.
104. Yimei Cai Xiaomin Yu Songnian Hu Jun, Y., *A Brief Review on the Mechanisms of miRNA Regulation.* 基因组蛋白质组与生物信息学报：英文版, 2009. **7**(4): p. 147-154.
105. Pillai, R.S., *MicroRNA function: multiple mechanisms for a tiny RNA?* RNA (New York, N.Y.), 2005. **11**(12): p. 1753-1761.
106. Patel, N., et al., *MicroRNAs can regulate human APP levels.* Molecular neurodegeneration, 2008. **3**: p. 10-1326-3-10.
107. Hebert, S.S., et al., *MicroRNA regulation of Alzheimer's Amyloid precursor protein expression.* Neurobiology of disease, 2009. **33**(3): p. 422-428.
108. Vilardo, E., et al., *MicroRNA-101 regulates amyloid precursor protein expression in hippocampal neurons.* The Journal of biological chemistry, 2010. **285**(24): p. 18344-18351.
109. Hébert, S.S., et al., *Loss of microRNA Cluster miR-29a/b-1 in Sporadic Alzheimer's Disease Correlates with Increased BACE1/β-Secretase Expression.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(17): p. 6415-6420.
110. Nunez-Iglesias, J., et al., *Joint genome-wide profiling of miRNA and mRNA expression in Alzheimer's disease cortex reveals altered miRNA regulation.* PloS one, 2010. **5**(2): p. e8898.
111. Wang, W.-X., et al., *Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter.* Acta neuropathologica, 2011. **121**(2): p. 193-205.
112. Ghanbari, M., et al., *Genome-wide identification of microRNA-related variants associated with risk of Alzheimer's disease.* Scientific reports 2016. **6**: p. 28387.

113. Crenshaw, E., et al., *Amyloid Precursor Protein Translation Is Regulated by a 3'UTR Guanine Quadruplex.* PloS one, 2015. **10**(11): p. e0143160.
114. Huppert, J.L. and S. Balasubramanian, *G-quadruplexes in promoters throughout the human genome.* Nucleic acids research, 2007. **35**(2): p. 406-413.
115. Huppert, J.L., et al., *G-quadruplexes: the beginning and end of UTRs.* Nucleic acids research, 2008. **36**(19): p. 6260-6268.
116. Thinakaran, G. and E.H. Koo, *Amyloid precursor protein trafficking, processing, and function.* The Journal of biological chemistry, 2008. **283**(44): p. 29615-29619.
117. Schedin-Weiss, S., B. Winblad, and L.O. Tjernberg, *The role of protein glycosylation in Alzheimer disease.* FEBS Journal, 2014. **281**(1): p. 46-62.
118. Tomita, S., Y. Kirino, and T. Suzuki, *Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism.* The Journal of biological chemistry, 1998. **273**(11): p. 6277-6284.
119. Tienari, P.J., et al., *The beta-amyloid domain is essential for axonal sorting of amyloid precursor protein.* The EMBO journal, 1996. **15**(19): p. 5218-5229.
120. Påhlsson, P., S.H. Shakin-Eshleman, and S.L. Spitalnik, *N-linked glycosylation of beta-amyloid precursor protein.* Biochemical and biophysical research communications, 1992. **189**(3): p. 1667-1673.
121. McFarlane, I., et al., *The role of the protein glycosylation state in the control of cellular transport of the amyloid β precursor protein.* Neuroscience, 1999. **90**(1): p. 15-25.
122. Akasaka-Manya, K., et al., *Increased bisecting and core-fucosylated N-glycans on mutant human amyloid precursor proteins.* Glycoconjugate Journal, 2008. **25**(8): p. 775-786.
123. Perdivara, I., et al., *Elucidation of O-glycosylation structures of the beta-amyloid precursor protein by liquid chromatography-mass spectrometry using electron transfer dissociation and collision induced dissociation.* Journal of proteome research, 2009. **8**(2): p. 631-642.
124. Hema Thanka Christlet, T. and K. Veluraja, *Database Analysis of O-Glycosylation Sites in Proteins.* Biophysical Journal, 2001. **80**(2): p. 952-960.
125. Haass, C., et al., *Amyloid beta-peptide is produced by cultured cells during normal metabolism.* Nature, 1992. **359**(6393): p. 322-325.
126. Shoji, M., et al., *Production of the Alzheimer Amyloid β Protein by Normal Proteolytic Processing.* Science, 1992. **258**(5079): p. 126-129.
127. Koo, E.H. and S.L. Squazzo, *Evidence that production and release of amyloid beta-protein involves the endocytic pathway.* Journal of Biological Chemistry, 1994. **269**(26): p. 17386.
128. Feyt, C., et al., *Phosphorylation of APP695 at Thr668 decreases γ-cleavage and extracellular Aβ.* Biochemical and Biophysical Research Communications, 2007. **357**(4): p. 1004-1010.
129. Serrano-Pozo, A., et al., *Neuropathological alterations in Alzheimer disease.* Cold Spring Harbor perspectives in medicine, 2011. **1**(1): p. a006189.
130. Bertram, L. and R.E. Tanzi, *Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses.* Nature reviews.Neuroscience, 2008. **9**(10): p. 768-778.
131. Hébert, S.S., et al., *Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes.* EMBO reports, 2006. **7**(7): p. 739-745.

132. Haass, C., *Take five--BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation.* The EMBO journal, 2004. **23**(3): p. 483-488.
133. Haass, C. and D.J. Selkoe, *Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide.* Nature reviews.Molecular cell biology, 2007. **8**(2): p. 101-112.
134. Zhang, Y.-w., et al., *Presenilin/gamma-secretase-dependent processing of beta-amyloid precursor protein regulates EGF receptor expression.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(25): p. 10613-10618.
135. Beckett, C., et al., *Nuclear signalling by membrane protein intracellular domains: the AICD enigma.* Cellular signalling, 2012. **24**(2): p. 402-409.
136. Zettl, H., et al., *Exploring the chemical space of gamma-secretase modulators.* Trends in pharmacological sciences, 2010. **31**(9): p. 402-410.
137. Nunan, J. and D.H. Small, *Regulation of APP cleavage by alpha-, beta- and gamma-secretases.* FEBS letters, 2000. **483**(1): p. 6-10.
138. Tanzi, R.E., *The synaptic Abeta hypothesis of Alzheimer disease.* Nature neuroscience, 2005. **8**(8): p. 977-979.
139. Granholm, A.C., et al., *ALzheimer's Disease and Down Syndrome: Developing a National Tissue Repository.* 2012(Journal, Electronic).
140. Rovelet-Lecrux, A., et al., *APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy.* Nature genetics, 2006. **38**(1): p. 24-26.
141. Weyer, S.W., et al., *Comparative analysis of single and combined APP/APLP knockouts reveals reduced spine density in APP-KO mice that is prevented by APPsalpha expression.* Acta neuropathologica communications, 2014. **2**(1): p. 36-5960-2-36.
142. Priller, C., et al., *Synapse formation and function is modulated by the amyloid precursor protein.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2006. **26**(27): p. 7212-7221.
143. Chatterjee, S. and J.K. Pal, *Role of 5'- and 3'-untranslated regions of mRNAs in human diseases.* Biology of the cell / under the auspices of the European Cell Biology Organization, 2009. **101**(5): p. 251-262.
144. Gray, N.K. and M. Wickens, *Control of translation initiation in animals.* Annual Review of Cell and Developmental Biology, 1998. **14**: p. 399.
145. Parker, R. and H. Song, *The enzymes and control of eukaryotic mRNA turnover.* Nature structural & molecular biology, 2004. **11**(2): p. 121-127.
146. Proudfoot, N.J., A. Furger, and M.J. Dye, *Integrating mRNA Processing with Transcription.* Cell, 2002. **108**(4): p. 501-512.
147. Wilkie, G.S., K.S. Dickson, and N.K. Gray, *Regulation of mRNA translation by 5'- and 3'-UTR-binding factors.* Trends in biochemical sciences, 2003. **28**(4): p. 182-188.
148. Ding, Y., et al., *In vivo genome-wide profiling of RNA secondary structure reveals novel regulatory features.* Nature, 2013.
149. Diwa, A., et al., *An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression.* Genes & development, 2000. **14**(10): p. 1249-1260.
150. Kozak, M., *Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(21): p. 8301-8305.
151. Millevoi, S., H. Moine, and S. Vagner, *G-quadruplexes in RNA biology.* Wiley interdisciplinary reviews.RNA, 2012. **3**(4): p. 495-507.

152. Kwok, C.K., M.E. Sherlock, and P.C. Bevilacqua, *Effect of loop sequence and loop length on the intrinsic fluorescence of G-quadruplexes.* Biochemistry, 2013. **52**(18): p. 3019-3021.
153. Biffi, G., et al., *Visualization and selective chemical targeting of RNA G-quadruplex structures in the cytoplasm of human cells.* Nature chemistry, 2014. **6**(1): p. 75-80.
154. Biffi, G., et al., *Quantitative visualization of DNA G-quadruplex structures in human cells.* Nature chemistry, 2013. **5**(3): p. 182-186.
155. Joachimi, A., A. Benz, and J.S. Hartig, *A comparison of DNA and RNA quadruplex structures and stabilities.* Bioorganic & medicinal chemistry, 2009. **17**(19): p. 6811-6815.
156. Huppert, J.L. and S. Balasubramanian, *Prevalence of quadruplexes in the human genome.* Nucleic acids research, 2005. **33**(9): p. 2908-2916.
157. Menendez, C., S. Frees, and P.S. Bagga, *QGRS-H Predictor: a web server for predicting homologous quadruplex forming G-rich sequence motifs in nucleotide sequences.* Nucleic acids research, 2012. **40**(Web Server issue): p. W96-W103.
158. Kwok, C.K., M.E. Sherlock, and P.C. Bevilacqua, *Decrease in RNA folding cooperativity by deliberate population of intermediates in RNA G-quadruplexes.* Angewandte Chemie (International ed.in English), 2013. **52**(2): p. 683-686.
159. Sosnick, T.R., *Characterization of tertiary folding of RNA by circular dichroism and urea.* Current protocols in nucleic acid chemistry / edited by Serge L.Beaucage ...[et al.], 2001. **Chapter 11**: p. Unit 11.5.
160. Kumari, S., et al., *An RNA G-quadruplex in the 5' UTR of the NRAS proto-oncogene modulates translation.* Nature chemical biology, 2007. **3**(4): p. 218-221.
161. Mullen, M.A., S.M. Assmann, and P.C. Bevilacqua, *Toward a digital gene response: RNA G-quadruplexes with fewer quartets fold with higher cooperativity.* Journal of the American Chemical Society, 2012. **134**(2): p. 812-815.
162. Subramanian, M., et al., *G-quadruplex RNA structure as a signal for neurite mRNA targeting.* EMBO reports, 2011. **12**(7): p. 697-704.
163. Lane, A.N., et al., *Stability and kinetics of G-quadruplex structures.* Nucleic acids research, 2008. **36**(17): p. 5482-5515.
164. Malgowska, M., et al., *Distinctive structural motifs of RNA G-quadruplexes composed of AGG, CGG and UGG trinucleotide repeats.* Nucleic acids research, 2014. **42**(15): p. 10196.
165. Ruttkay-Nedecky, B., et al., *G-quadruplexes as sensing probes.* Molecules (Basel, Switzerland), 2013. **18**(12): p. 14760-14779.
166. Mullen, M.A., et al., *RNA G-Quadruplexes in the model plant species Arabidopsis thaliana: prevalence and possible functional roles.* Nucleic acids research, 2010. **38**(22): p. 8149-8163.
167. Beaudoin, J.D., R. Jodoin, and J.P. Perreault, *In-line probing of RNA G-quadruplexes.* Methods (San Diego, Calif.), 2013.
168. Morris, M.J. and S. Basu, *An unusually stable G-quadruplex within the 5'-UTR of the MT3 matrix metalloproteinase mRNA represses translation in eukaryotic cells.* Biochemistry, 2009. **48**(23): p. 5313-5319.
169. Culvenor, J.G., et al., *Expression of the Amyloid Precursor Protein of Alzheimer's Disease on the Surface of Transfected HeLa Cells.* Experimental cell research, 1995. **220**(2): p. 474-481.
170. Weidemann, A., et al., *Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein.* Cell, 1989. **57**(1): p. 115-126.

171. Dieterich, D.C., et al., *Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT).* Proc Natl Acad Sci U S A, 2006. **103**(25): p. 9482-7.
172. Beaudoin, J.D. and J.P. Perreault, *5'-UTR G-quadruplex structures acting as translational repressors.* Nucleic acids research, 2010. **38**(20): p. 7022-7036.
173. Bugaut, A. and S. Balasubramanian, *5'-UTR RNA G-quadruplexes: translation regulation and targeting.* Nucleic acids research, 2012. **40**(11): p. 4727-4741.
174. Endoh, T., Y. Kawasaki, and N. Sugimoto, *Translational halt during elongation caused by G-quadruplex formed by mRNA.* Methods (San Diego, Calif.), 2013. **64**(1): p. 73-78.
175. Endoh, T., Y. Kawasaki, and N. Sugimoto, *Stability of RNA quadruplex in open reading frame determines proteolysis of human estrogen receptor alpha.* Nucleic acids research, 2013. **41**(12): p. 6222-6231.
176. Arora, A. and B. Suess, *An RNA G-quadruplex in the 3' UTR of the proto-oncogene PIM1 represses translation.* RNA biology, 2011. **8**(5): p. 802-805.
177. Beaudoin, J.D. and J.P. Perreault, *Exploring mRNA 3'-UTR G-quadruplexes: evidence of roles in both alternative polyadenylation and mRNA shortening.* Nucleic acids research, 2013. **41**(11): p. 5898-5911.
178. Lammich, S., et al., *Translational repression of the disintegrin and metalloprotease ADAM10 by a stable G-quadruplex secondary structure in its 5'-untranslated region.* The Journal of biological chemistry, 2011. **286**(52): p. 45063-45072.
179. Fisette, J.F., et al., *A G-rich element forms a G-quadruplex and regulates BACE1 mRNA alternative splicing.* Journal of neurochemistry, 2012. **121**(5): p. 763-773.
180. Kumari, S., et al., *An RNA G-quadruplex in the 5' UTR of the NRAS proto-oncogene modulates translation.* Nat Chem Biol, 2007. **3**(4): p. 218-21.
181. Shahid, R., A. Bugaut, and S. Balasubramanian, *The BCL-2 5' untranslated region contains an RNA G-quadruplex-forming motif that modulates protein expression.* Biochemistry, 2010. **49**(38): p. 8300-6.
182. Arora, A. and B. Suess, *An RNA G-quadruplex in the 3' UTR of the proto-oncogene PIM1 represses translation.* RNA Biol, 2011. **8**(5): p. 802-5.
183. Arora, A., et al., *Inhibition of translation in living eukaryotic cells by an RNA G-quadruplex motif.* RNA, 2008. **14**(7): p. 1290-6.
184. Kwok, C.K., et al., *A stable RNA G-quadruplex within the 5'-UTR of Arabidopsis thaliana ATR mRNA inhibits translation.* Biochem J, 2015. **467**(1): p. 91-102.
185. Millevoi, S., H. Moine, and S. Vagner, *G-quadruplexes in RNA biology.* Wiley Interdiscip Rev RNA, 2012. **3**(4): p. 495-507.
186. Brazda, V., et al., *DNA and RNA quadruplex-binding proteins.* Int J Mol Sci, 2014. **15**(10): p. 17493-517.
187. Subaran, S.S., et al., *hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies.* Nature Structural & Molecular Biology, 2010. **17**(6): p. 732-739.
188. Yang, Y.C., et al., *CLIPdb: a CLIP-seq database for protein-RNA interactions.* BMC Genomics, 2015. **16**: p. 51.
189. Stefanovic, S., et al., *Fragile X mental retardation protein interactions with a G quadruplex structure in the 3'-untranslated region of NR2B mRNA.* Mol Biosyst, 2015.
190. Lagier-Tourenne, C., et al., *Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs.* Nat Neurosci, 2012. **15**(11): p. 1488-97.
191. Westmark, C.J. and J.S. Malter, *The regulation of AbetaPP expression by RNA-binding proteins.* Ageing Res Rev, 2012. **11**(4): p. 450-9.

192. Donev, R., et al., *A role for SC35 and hnRNPA1 in the determination of amyloid precursor protein isoforms.* Mol Psychiatry, 2007. **12**(7): p. 681-90.
193. von Hacht, A., et al., *Identification and characterization of RNA guanine-quadruplex binding proteins.* Nucleic Acids Res, 2014. **42**(10): p. 6630-44.
194. Bugaut, A. and S. Balasubramanian, *5'-UTR RNA G-quadruplexes: translation regulation and targeting.* Nucleic Acids Res, 2012. **40**(11): p. 4727-41.
195. Woo, H.H., et al., *Nucleolin mediates microRNA-directed CSF-1 mRNA deadenylation but increases translation of CSF-1 mRNA.* Mol Cell Proteomics, 2013. **12**(6): p. 1661-77.
196. Stefanovic, S., G.J. Bassell, and M.R. Mihailescu, *G quadruplex RNA structures in PSD-95 mRNA: potential regulators of miR-125a seed binding site accessibility.* RNA, 2015. **21**(1): p. 48-60.
197. Oyama, F., et al., *Down's syndrome: up-regulation of beta-amyloid protein precursor and tau mRNAs and their defective coordination.* Journal of neurochemistry, 1994. **62**(3): p. 1062.
198. Matsui, T., et al., *Expression of APP pathway mRNAs and proteins in Alzheimer's disease.* Brain research, 2007. **1161**: p. 116.
199. Head, E. and I.T. Lott, *Down syndrome and beta-amyloid deposition.* Current opinion in neurology, 2004. **17**(2): p. 95-100.
200. Sleegers, K., et al., *APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy.* Brain : a journal of neurology, 2006. **129**(Pt 11): p. 2977-2983.
201. Haass, C., et al., *The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway.* Nature medicine, 1995. **1**(12): p. 1291.
202. Shin, J.-Y., et al., *Swedish mutation within amyloid precursor protein modulates global gene expression towards the pathogenesis of Alzheimer's disease.* BMB Reports, 2010. **43**(10): p. 704-709.
203. Glisovic, T., et al., *RNA-binding proteins and post-transcriptional gene regulation.* FEBS Letters, 2008. **582**(14): p. 1977-1986.
204. Vogel, C. and E.M. Marcotte, *Insights into the regulation of protein abundance from proteomic and transcriptomic analyses.* NATURE REVIEWS GENETICS, 2012. **13**(4): p. 227-232.
205. Greenbaum, D., et al., *Comparing protein abundance and mRNA expression levels on a genomic scale.* GENOME BIOLOGY, 2003. **4**(9): p. 117-117.
206. Le Quesne, J.P.C., et al., *Dysregulation of protein synthesis and disease.* JOURNAL OF PATHOLOGY, 2010. **220**(2): p. 140-151.
207. Sonenberg, N., H. Imataka, and A. Gradi, *A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation.* The EMBO Journal, 1998. **17**(24): p. 7480-7489.
208. Sonenberg, N. and A.G. Hinnebusch, *Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets.* Cell, 2009. **136**(4): p. 731-745.
209. Gebauer, F. and M.W. Hentze, *Molecular mechanisms of translational control.* Nature Reviews Molecular Cell Biology, 2004. **5**(10): p. 827-835.
210. Richter, J.D. and N. Sonenberg, *Regulation of cap-dependent translation by eIF4E inhibitory proteins.* Nature, 2005. **433**(7025): p. 477-480.
211. Pesole, G., et al., *Structural and functional features of eukaryotic mRNA untranslated regions.* Gene, 2001. **276**(1): p. 73-81.
212. Gebauer, F., T. Preiss, and M.W. Hentze, *From cis-regulatory elements to complex RNPs and back.* Cold Spring Harbor perspectives in biology, 2012. **4**(7): p. a012245.

213. Szostak, E. and F. Gebauer, *Translational control by 3'-UTR-binding proteins.* Briefings in functional genomics, 2013. **12**(1): p. 58.
214. Licatalosi, D.D. and R.B. Darnell, *Resolving RNA complexity to decipher regulatory rules governing biological networks.* Nature reviews. Genetics, 2010. **11**(1): p. 75-87.
215. Mazumder, B., V. Seshadri, and P.L. Fox, *Translational control by the 3'-UTR: the ends specify the means.* Trends in Biochemical Sciences, 2003. **28**(2): p. 91-98.
216. Pesole, G., et al., *UTRdb and UTRsite: specialized databases of sequences and functional elements of 5' and 3' untranslated regions of eukaryotic mRNAs. Update 2002.* Nucleic acids research, 2002. **30**(1): p. 335-340.
217. Di Giammartino, Dafne C., K. Nishida, and James L. Manley, *Mechanisms and Consequences of Alternative Polyadenylation.* Molecular Cell, 2011. **43**(6): p. 853-866.
218. Yarchoan, R., et al., *Response of human-immunodeficiency-virus-associated neurological disease to 3'-azido-3'-deoxythymidine.* Lancet (London, England), 1987. **1**(8525): p. 132.
219. Erard, M.S., et al., *A major nucleolar protein, nucleolin, induces chromatin decondensation by binding to histone Hl.* European Journal of Biochemistry, 1988. **175**(3): p. 525-530.
220. Yang, T.H., et al., *Purification and characterization of nucleolin and its identification as a transcription repressor.* Molecular and Cellular Biology, 1994. **14**(9): p. 6068-6074.
221. Bugler, B., et al., *RNA binding fragments from nucleolin contain the ribonucleoprotein consensus sequence.* Journal of Biological Chemistry, 1987. **262**(23): p. 10922.
222. Westmark, C.J. and J.S. Malter, *Up-regulation of Nucleolin mRNA and Protein in Peripheral Blood Mononuclear Cells by Extracellular-regulated Kinase.* Journal of Biological Chemistry, 2001. **276**(2): p. 1119-1126.
223. Dreyfuss, G. and S. Piñol-Roma, *Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm.* Nature, 1992. **355**(6362): p. 730-732.
224. Görlach, M., C.G. Burd, and G. Dreyfuss, *The determinants of RNA-binding specificity of the heterogeneous nuclear ribonucleoprotein C proteins.* Journal of Biological Chemistry, 1994. **269**(37): p. 23074.
225. Westmark, C.J. and J.S. Malter, *Extracellular-regulated kinase controls β-amyloid precursor protein mRNA decay.* Molecular Brain Research, 2001. **90**(2): p. 193-201.
226. Kang, M.-J., et al., *HuD regulates coding and noncoding RNA to induce APP→Aβ processing.* Cell reports, 2014. **7**(5): p. 1401-1409.
227. Deschênes-Furry, J., N. Perrone-Bizzozero, and B.J. Jasmin, *The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity.* BioEssays, 2006. **28**(8): p. 822-833.
228. Pascale, A., M. Amadio, and A. Quattrone, *Defining a neuron: neuronal ELAV proteins.* Cellular and Molecular Life Sciences, 2008. **65**(1): p. 128-140.
229. Kim, V.N., *MicroRNA biogenesis: coordinated cropping and dicing.* Nature Reviews Molecular Cell Biology, 2005. **6**(5): p. 376-385.
230. Peters, L. and G. Meister, *Argonaute Proteins: Mediators of RNA Silencing.* Molecular Cell, 2007. **26**(5): p. 611-623.
231. Hébert, S.S., et al., *MicroRNA regulation of Alzheimer's Amyloid precursor protein expression.* Neurobiology of Disease, 2009. **33**(3): p. 422-428.
232. Long, J.M., B. Ray, and D.K. Lahiri, *MicroRNA-153 physiologically inhibits expression of amyloid-β precursor protein in cultured human fetal brain cells and is dysregulated in a subset of Alzheimer disease patients.* The Journal of biological chemistry, 2012. **287**(37): p. 31298-31310.

233. Delay, C., et al., *Alzheimer-specific variants in the 3'UTR of Amyloid precursor protein affect microRNA function.* Molecular neurodegeneration, 2011. **6**(1): p. 70-70.
234. Chen, Z., et al., *The biological functions of G-quadruplex.* Sheng li ke xue jin zhan [Progress in physiology], 2010. **41**(5): p. 329-334.
235. Woo, H.-H., et al., *Nucleolin mediates microRNA-directed CSF-1 mRNA deadenylation but increases translation of CSF-1 mRNA.* Molecular & cellular proteomics : MCP, 2013. **12**(6): p. 1661-1677.
236. Stefanovic, S., G.J. Bassell, and M.R. Mihailescu, *G quadruplex RNA structures in PSD-95 mRNA: potential regulators of miR-125a seed binding site accessibility.* RNA (New York, N.Y.), 2015. **21**(1): p. 48-60.
237. Priller, C., et al., *Synapse Formation and Function Is Modulated by the Amyloid Precursor Protein.* Journal of Neuroscience, 2006. **26**(27): p. 7212-7221.
238. Walsh, D.M. and D.J. Selkoe, *Deciphering the Molecular Basis of Memory Failure in Alzheimer's Disease*. 2004, Elsevier Inc: United States. p. 181-193.
239. Sisodia, S., et al., *Amyloid beta from axons and dendrites reduces local spine number and plasticity.* Nature Neuroscience, 2010. **13**(2): p. 190-196.
240. Rabe, S., et al., *The Swedish APP mutation alters the effect of genetically reduced BACE1 expression on the APP processing.* Journal of Neurochemistry, 2011. **119**(1): p. 231-239.
241. Liu, F.-L., T.-Y. Liu, and F.-L. Kung, *FKBP12 regulates the localization and processing of amyloid precursor protein in human cell lines.* Journal of Biosciences, 2014. **39**(1): p. 85-95.
242. Robakis, N.K., et al., *Chromosome 21q21 sublocalisation of gene encoding beta-amyloid peptide in cerebral vessels and neuritic (senile) plaques of people with Alzheimer disease and Down syndrome.* Lancet, 1987. **1**(8529): p. 384-385.
243. Granholm, A.C., et al. *Alzheimer's Disease and Down Syndrome: Developing a National Tissue Repository*. 2012.
244. Ribeiro, M.M., et al., *G-quadruplex formation enhances splicing efficiency of PAX9 intron 1.* Human Genetics, 2015. **134**(1): p. 37-44.
245. Morris, M.J., et al., *An RNA G-quadruplex is essential for cap-independent translation initiation in human VEGF IRES.* Journal of the American Chemical Society, 2010. **132**(50): p. 17831-17839.
246. Hacht, A., et al., *Identification and characterization of RNA guanine-quadruplex binding proteins.* Nucleic acids research, 2014. **42**(10): p. 6630-6644.
247. Zhang, Y., et al., *FMRP interacts with G-quadruplex structures in the 3 '-UTR of its dendritic target Shank1 mRNA.* RNA BIOLOGY, 2014. **11**(11): p. 1364-1374.
248. Vasilyev, N., et al., *Crystal structure reveals specific recognition of a G-quadruplex RNA by a β-turn in the RGG motif of FMRP.* Proceedings of the National Academy of Sciences, 2015. **112**(39): p. E5391-E5400.
249. Kenny, P. and S. Ceman, *RNA Secondary Structure Modulates FMRP's Bi-Functional Role in the MicroRNA Pathway.* International Journal of Molecular Sciences, 2016. **17**(6): p. 985.
250. Ruberti, F., C. Barbato, and C. Cogoni, *Post-transcriptional regulation of amyloid precursor protein by microRNAs and RNA binding proteins.* Communicative & integrative biology, 2010. **3**(6): p. 499-503.
251. Jin, P. and S.T. Warren, *Understanding the molecular basis of fragile X syndrome.* Human molecular genetics, 2000. **9**(6): p. 901-908.

252. Verkerk, A.J.M.H., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome.* Cell, 1991. **65**(5): p. 905-914.
253. Siomi, H., et al., *The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein.* Cell, 1993. **74**(2): p. 291-298.
254. Darnell, J.C., et al., *Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes.* Genes & development, 2005. **19**(8): p. 903-918.
255. Kuryavyi, V., et al., *Structure-function studies of FMRP RGG peptide recognition of an RNA duplex-quadruplex junction.* Nature Structural & Molecular Biology, 2011. **18**(7): p. 796-804.
256. Darnell, J.C., et al., *Discrimination of common and unique RNA-binding activities among Fragile X mental retardation protein paralogs.* Human Molecular Genetics, 2009. **18**(17): p. 3164-3177.
257. Zhang, Y., et al., *The fragile X mental retardation syndrome protein interacts with novel homologs FXR1 and FXR2.* The EMBO journal, 1995. **14**(21): p. 5358-5366.
258. Agulhon, C., et al., *Expression of FMR1, FXR1, and FXR2 Genes in Human Prenatal Tissues.* Journal of Neuropathology and Experimental Neurology, 1999. **58**(8): p. 867-880.
259. Siomi, M.C., et al., *Specific sequences in the fragile X syndrome protein FMR1 and the FXR proteins mediate their binding to 60S ribosomal subunits and the interactions among them.* Molecular and Cellular Biology, 1996. **16**(7): p. 3825-3832.
260. Zhang, J., et al., *Fragile X-Related Proteins Regulate Mammalian Circadian Behavioral Rhythms.* The American Journal of Human Genetics, 2008. **83**(1): p. 43-52.
261. Lumaban, J.G. and D.L. Nelson, *The Fragile X proteins Fmrp and Fxr2p cooperate to regulate glucose metabolism in mice.* Human molecular genetics, 2015. **24**(8): p. 2175-2184.
262. Huang, D.W., R.A. Lempicki, and B.T. Sherman, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nature Protocols, 2008. **4**(1): p. 44-57.
263. Yang, Y.C.T., et al., *CLIPdb: a CLIP-seq database for protein-RNA interactions.* BMC GENOMICS, 2015. **16**(1): p. 51-51.
264. Ramos, A., D. Hollingworth, and A. Pastore, *G-quartet-dependent recognition between the FMRP RGG box and RNA.* RNA (New York, N.Y.), 2003. **9**(10): p. 1198-1207.
265. Phan, A.T., et al., *Structure-function studies of FMRP RGG peptide recognition of an RNA duplex-quadruplex junction.* Nature structural & molecular biology, 2011. **18**(7): p. 796-804.
266. Napoli, I., et al., *The Fragile X Syndrome Protein Represses Activity-Dependent Translation through CYFIP1, a New 4E-BP.* Cell, 2008. **134**(6): p. 1042-1054.
267. Ishizuka, A., M.C. Siomi, and H. Siomi, *A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins.* Genes & development, 2002. **16**(19): p. 2497-2508.
268. Muddashetty, Ravi S., et al., *Reversible Inhibition of PSD-95 mRNA Translation by miR-125a, FMRP Phosphorylation, and mGluR Signaling.* Molecular Cell, 2011. **42**(5): p. 673-688.
269. Chen, E., et al., *Fragile X Mental Retardation Protein Regulates Translation by Binding Directly to the Ribosome.* Molecular Cell, 2014. **54**(3): p. 407-417.

270. Musiek, E.S., D.D. Xiong, and D.M. Holtzman, *Sleep, circadian rhythms, and the pathogenesis of Alzheimer disease.* Experimental & molecular medicine, 2015. **47**: p. e148.
271. Peter-Derex, L., et al., *Sleep and Alzheimer's disease.* SLEEP MEDICINE REVIEWS, 2015. **19**: p. 29-38.
272. Roh, J.H., et al., *Disruption of the sleep-wake cycle and diurnal fluctuation of β-amyloid in mice with Alzheimer's disease pathology.* Science translational medicine, 2012. **4**(150): p. 150ra122.
273. Schneider, F., et al., *Behavioral and EEG changes in male 5xFAD mice.* Physiology & behavior, 2014. **135**: p. 25-33.
274. Ju, Y.E.S., B.P. Lucey, and D.M. Holtzman, *Sleep and Alzheimer disease pathology-a bidirectional relationship.* NATURE REVIEWS NEUROLOGY, 2014. **10**(2): p. 115-119.
275. Spira, A.P., et al., *Self-reported Sleep and β-Amyloid Deposition in Community-Dwelling Older Adults.* JAMA Neurology, 2013. **70**(12): p. 1537-1543.
276. Gonzalez, V., et al., *Identification and Characterization of Nucleolin as a c-myc G-quadruplex-binding Protein.* Journal of Biological Chemistry, 2009. **284**(35): p. 23622-23635.
277. Takahama, K. and T. Oyoshi, *Specific Binding of Modified RGG Domain in TLS/FUS to G-Quadruplex RNA: Tyrosines in RGG Domain Recognize 2 '-OH of the Riboses of Loops in G-Quadruplex.* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, 2013. **135**(48): p. 18016-18019.
278. Basundra, R., et al., *A novel G-quadruplex motif modulates promoter activity of human thymidine kinase 1.* FEBS Journal, 2010. **277**(20): p. 4254-4264.
279. Martadinata, H. and A.T. Phan, *Structure of Human Telomeric RNA (TERRA): Stacking of Two G-Quadruplex Blocks in K+ Solution.* BIOCHEMISTRY, 2013. **52**(13): p. 2176-2183.
280. Valencia-Cruz, G., et al., *Kbg and Kv1.3 channels mediate potassium efflux in the early phase of apoptosis in Jurkat T lymphocytes.* American Journal of Physiology - Cell Physiology, 2009. **297**(6): p. 1544-1553.
281. Hardin, C.C., et al., *CATION-DEPENDENT TRANSITION BETWEEN THE QUADRUPLEX AND WATSON-CRICK HAIRPIN FORMS OF D(CGCG3GCG).* BIOCHEMISTRY, 1992. **31**(3): p. 833-841.
282. Humphries, E.S.A. and C. Dart, *Neuronal and Cardiovascular Potassium Channels as Therapeutic Drug Targets: Promise and Pitfalls.* Journal of biomolecular screening, 2015. **20**(9): p. 1055-1073.
283. Deng, P.-Y., et al., *FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels.* Neuron, 2013. **77**(4): p. 696-711.
284. Latorre, R., et al., *Varieties of calcium-activated potassium channels.* Annual review of physiology, 1989. **51**: p. 385.
285. Yang, X., et al., *Potassium-sensitive G-quadruplex DNA for sensitive visible potassium detection.* ANALYST, 2010. **135**(1): p. 71-75.
286. Liu, H.H., et al., *RNA G-quadruplex formation in defined sequence in living cells detected by bimolecular fluorescence complementation.* CHEMICAL SCIENCE, 2016. **7**(7): p. 4573-4581.
287. Stefanovic, S., G.J. Bassell, and M.R. Mihailescu, *G quadruplex RNA structures in PSD-95 mRNA: potential regulators of miR-125a seed binding site accessibility.* RNA-A PUBLICATION OF THE RNA SOCIETY, 2015. **21**(1): p. 48-60.

288. Sontag, E., et al., *Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology.* Journal of neuropathology and experimental neurology, 2004. **63**(4): p. 287.
289. Fisette, J.-F., et al., *A G-Rich element forms a G-quadruplex and regulates BACE1 mRNA alternative splicing: G-rich element regulates BACE1 alternative splicing.* Journal of Neurochemistry, 2012. **121**(5): p. 763-773.
290. Lammich, S., et al., *Translational repression of the disintegrin and metalloprotease ADAM10 by a stable G-quadruplex secondary structure in its 5'-untranslated region.* The Journal of biological chemistry, 2011. **286**(52): p. 45063-45072.
291. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics.* Science, 2002. **297**(5580): p. 353-6.
292. Yoon, S.-S. and S.A. Jo, *Mechanisms of Amyloid-β Peptide Clearance: Potential Therapeutic Targets for Alzheimer's Disease.* Biomolecules & therapeutics, 2012. **20**(3): p. 245.

Curriculum Vitae

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Profile: I am a highly motivated individual and very passionate about the field of biological science. Having a concentration in molecular biology, I have learned cutting-edge technical approaches that have allowed me to investigate biological mechanisms. I have also demonstrated organizing skills, a firm sense of responsibility, and capacity to work hard under pressure. I have also been able to train students with no experience in molecular biology and guide them to learn lab techniques and skills in order to be able to work in a laboratory. I have a passion for science and science education as demonstrated by the various internships, mentorship, and awards outlined in this curriculum vitae

Education:

Graduate Institution: Location:
Drexel University (2010-2016) Philadelphia, PA
Degree: Ph.D. Biology
Dissertation Title: *Characterizing G-quadruplex Mediated Regulation of APP Translation*

Undergraduate Institution: Location:
Cheyney University (2006-2010) Cheyney, PA
Degree*: Bachelor of Arts*
Honors: *Summa Cum Laude*

Research Experience:

Drexel University Philadelphia, PA (2010-2016)
Graduate Research Assistant to Dr. Aleister Saunders
The research of this lab is to understand how the amyloid precursor protein (APP) is processed into peptides that cause Alzheimer's disease using cutting-edge molecular and genetic tools. My research is aimed at describing how a novel RNA G-quadruplex sequence in the 3' untranslated region of APP regulates its translation.

Skills/Techniques:

Aseptic Technique, Western Blot, Cell culture (HEK 293 cells, PC12 cells, Rat Cortical Neurons), DNA/RNA Extraction/Purification, RT-PCR, SDS PAGE, Transformation, Cloning, Transfection of plasmid DNA, Dual Luciferase Assay, Cell Culture Medium preparation, Immunoprecipitation, Bioorthogonal Non-canonical Amino acid Tagging (BONCAT), Silver Staining, some Microscopy.

USDA Eastern Regional Research Center Wyndmoor, PA (2009-2010)

Research Lab Technician to Dr. Kathleen Rajkowski

With funds granted by the USDA to Cheyney University, I investigated the presence of pathogenic microorganisms associated with foreign and domestic catfish. I was responsible for testing and identifying those microorganisms with various techniques used in microbiology. As a lab technician, I was responsible for training and teaching new members on lab procedures and protocols.

Skills/ Techniques:

Aerobic Plate Count, *E. coli* Coliform Count, MPN, Medium and Buffer Preparations

Los Alamos National Laboratory Los Alamos, NM (2008, 2009)

Research Lab Assistant to Dr. Chang Yub-Kim

I had the opportunity to spend two summer internships at the Los Alamos National Laboratory where I was involved in the purification of proteins associated with bacterium *Mycobacterium tuberculosis,* which is the organism that causes the disease Tuberculosis. The proteins that I purified were from the bacterium that was resistant to many types of antibiotics.

Skills/ Techniques:

Protein Purification, DNA agarose gels, SDS PAGE, DNA/RNA Extraction.

Wistar Institute Philadelphia, PA (2007)

Research Lab Assistant to Dr. Andrew Caton

My responsibility at the Wistar Institute was to determine IC50 for transduction of the PR8 influenza virus through the use of Hemagglutinin Assays. As this was my first research internship, my other responsibilities were learning how to maintain and culture cells and work aseptically.

Skills/Techniques:

Aseptic Technique, Cell Culture (MDCK cells)

Awards:

2015: Summer Program in Neuroscience, Ethics, and Survival (SPINES) Participant

2015: Drexel University International Travel Award

2014-Current: NIH/NIA Aging Research Dissertation Award to Increase Diversity (R36)

2014-Current: Society for Neuroscience- Neuroscience Scholars Program Associate

2012-2014: National Science Foundation GK-12 Fellowship

2010-2012: National Science Foundation LSAMP Bridge to the Doctorate Fellowship
2010: Nominated for the Ali-Zaidi Award for Excellence
2009: United Negro College Fund Amtrak travel Award
2009: Siemens Scholar for providing service in the STEM discipline at Guion S. Bluford Elementary School
2009: HBCU-Up Conference 1st place winner in Biology
2009: President's Award for Academic Excellence
2007-2008, 2010: Dean's List

Publications:

Crenshaw E, Leung BP, Kwok CK, Sharoni M, Olson K, Sebastian NP, et al. (2015) Amyloid Precursor Protein Translation Is Regulated by a 3'UTR Guanine Quadruplex. PLoS ONE 10(11): e0143160. doi:10.1371/journal.pone.0143160

Professional Conferences: Presenter

Ezekiel Crenshaw, Michael Akins, Aleister Saunders "The Amyloid Precursor Protein is Negatively Regulated by a 3'UTR G-quadruplex" Society for Neuroscience 2015

Ezekiel Crenshaw, Michael Akins, Aleister Saunders "The Amyloid Precursor Protein is Negatively Regulated by a 3'UTR G-quadruplex" 5th International Meeting on Quadruplex Nucleic Acids Bordeaux, France 2015

Ezekiel Crenshaw, Aleister Saunders "A Guanine Quadruplex Negatively Regulates Amyloid Precursor Protein Expression" Society for Neuroscience 2014

Ezekiel Crenshaw, Aleister Saunders "A Guanine Quadruplex Negatively Regulates Amyloid Precursor Protein Expression" Drexel University Research Day 2014

Ezekiel Crenshaw, Aleister Saunders "Understanding Amyloid Precursor Protein through mRNA Transport" 14th Annual Philadelphia AMP Research Symposium and Mentoring Conference 2011

Ezekiel Crenshaw, Chang Yub-Kim "Improvement in the Purification of *Mycobacterium Tuberculosis"* Historically Black Colleges and Universities Undergraduate Program (HBCU-UP) 2009 National Research Conference, Washington, D.C 2009 (1st place in Biological Science)

Ezekiel Crenshaw, Chang Yub-Kim "Improvement in the Purification of *Mycobacterium Tuberculosis*" DOE Science and Research Challenge. Oak Ridge National Laboratory, Oak Ridge, TN, November 2009

Ezekiel Crenshaw, Chang Yub-Kim "Purification of *Mycobacterium Tuberculosis"* Historically Black Colleges and Universities Undergraduate Program (HBCU-UP) 2008

Ezekiel Crenshaw, Chang Yub-Kim "Purification of *Mycobacterium Tuberculosis*" DOE Science and Research Challenge. Oak Ridge National Laboratory, Oak Ridge, TN, November 2008

Professional Conferences: Attended

2015: Summer Program in Neuroscience, Ethics, and Survival (SPINES) Course Participant

2012: Emerging Researchers National Conference
2012: NSF Joint Annual Meeting Conference
2012: Alzheimer's Disease Research Summit

Activities:
2014-Current: Society for Neuroscience member
2010- Current: Biology Graduate Student Association
2009-Current: Alpha Phi Alpha Fraternity, Inc.
2009-2010: Beta Kappa Chi Scientific Honors Society

Teaching/Mentorship:

2016-Current: Math 200 Instructor University of Pennsylvania Veterans Upward Bound Program: I am currently the Math 200 instructor for the University of Pennsylvania's Veterans Upward Bound Program. My role is to prepare veterans with the skills needed to take a college math entrance exam. Since the students have served in various capacities within the U.S armed forces, their services have taken them away from school for various years. In the Math 200 course, I am covering topics such as order of operations, the real number system, and will end the course with an introduction to algebra. I have developed a syllabus for this course, lesson plans, homework assignments, quizzes/exams, and presentations for the students to practice public speaking.

2013-2014 Girard Academic Music Program- Returning as a fellow in the NSF GK-12 Program, I worked with 11th and 12 grade students in Environmental Science class through a combination of learning modules and hands-on science activities. My responsibility was to connect the content that the students learned in class with challenges that scientist face in order to show how engineers help to overcome those challenges. Since I was working with seniors this time, I was able to provide insight to the students about applying to colleges and universities for those interested in STEM.

2012-2013 Science Leadership Academy- As a fellow in the NSF GK-12 project I teach engineering and science to 9th graders in the Biochemistry of Watersheds class. Through a combination learning modules and hands-on science activities, I am connecting the content that the students learn in class with challenges that scientist face in order to show how engineers help to overcome those challenges. I will also have the opportunity to travel to Kenya where I will conduct the same activities for the students at the Alliance High School in Nairobi. This mentoring opportunity enabled me to help the students become interested in science or engineering.